Osteocyte Apoptosis and Bone Adaptation

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Osteocyte Apoptosis and Bone Adaptation

Sian Djien Tan

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CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Introduction

Bone is a living tissue and is able to alter its mass and structure to its mechanical environment. Increased mechanical loading results in a gain of bone mass and mineral density *in vivo*[1-3], while unloading of bones is known to reduce bone mass, mineral content, and bone matrix protein production[4,5]. This process is known as functional adaptation of bone, and it serves to obtain bones that combine a proper resistance against mechanical failure with a minimum use of material[6,7]. Adaptation of bone to changing environmental demands is obtained during the complicated process of bone remodeling[2,8-10]. Orthodontic tooth movement is an example of clinically relevant functional adaptation of bone. After application of an orthodontic load, osteoclast recruitment results in bone resorption at the site clinically referred to as "pressure side", while osteoblast recruitment results in bone formation at the site clinically referred to as "tension side"[11,12] (Figure 1.1). To understand the process of bone adaptation at the macroscopic level, more information is needed on the cellular background underlying this process of bone adaptation.

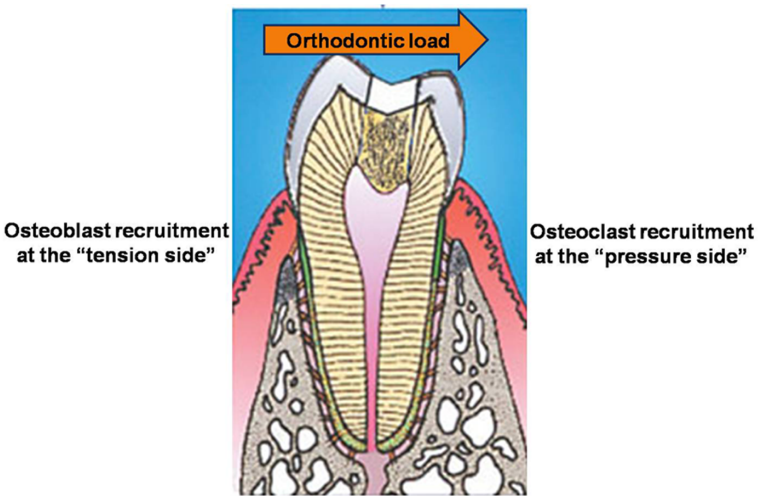

Figure 1.1 *Illustration of an orthodontic tooth movement. After application of an orthodontic load, osteoclast recruitment results in bone resorption at the site clinically referred to as "pressure side", while osteoblast recruitment results in bone formation at the site clinically referred to as "tension side".*

The role of osteocytes in mechanical adaptation of bone

Osteocytes are the predominant bone cells and considered to be the orchestrators of bone adaptation to loading[8,10,13-16]. Osteocytes are surrounded by bone, and are in contact with neighboring osteocytes via long slender cell processes, located in canaliculi, which are filled with pericellular interstitial fluid[17,18]. These cell processes not only contact neighboring osteocytes, but also cells at the bone surface such as osteoblasts, bone lining cells, and osteoclasts[19]. This three-dimensional network of interconnected cells is present throughout bone, and it has been suggested that this osteocyte network with its accompanying lacuno-canalicular porosity is the site of mechanosensing in bone[8,14,16,20-22].

How the osteocytes sense mechanical loading via this extensive three-dimensional network is explained via the proposed canalicular flow hypothesis[8,23] (Figure 1.2). When bone is loaded, interstitial fluid

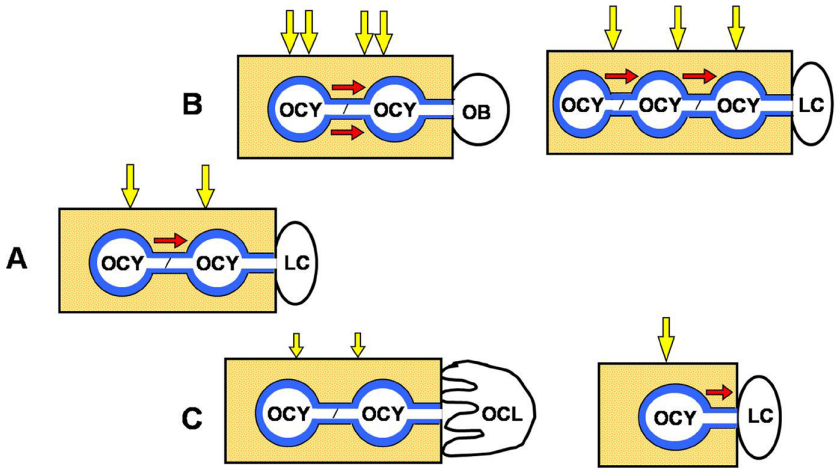

Figure 1.2 *Schematic representation of the canalicular flow hypothesis. (A) In the steady state, normal mechanical use ensures a basal level of fluid flow through the lacuno-canalicular porosity. This basal flow keeps the osteocytes viable and also ensures basal osteocyte activation and signaling, thereby suppressing osteoblastic activity as well as osteoclastic attack. (B) During (local) overuse, the osteocytes are over-activated by enhanced fluid flow, leading to osteoblast-recruiting signals. Subsequent osteoblastic bone formation reduces the overuse until normal mechanical use is re-established, thereby re-establishing the steady state of basal fluid flow. (C) During (local) disuse, the osteocytes are inactivated by lack of fluid flow. Inactivation either leads to release of osteoclast-recruiting signals or to lack of osteoclast suppressing signals, or both. Subsequent osteoclastic bone resorption re-establishes normal mechanical use and basal fluid flow. OCY, osteocyte; LC, lining cell; OB, osteoblast; OCL osteoclast; yellow arrows, mechanical use; red arrows, fluid flow.*

is squeezed through the three-dimensional network, resulting in a fluid flow. This flow results in a strain-driven movement of interstitial fluid, through the canaliculi and along the osteocyte processes, which is sensed and transduced by osteocytes[14,16,24,25]. Upon this flow of interstitial fluid osteocytes subsequently produce signaling molecules stimulating osteoclasts to resorb bone, or osteoblasts to build new bone[26]. Mechanotransduction then includes the translation of canalicular flow by osteocytes into cell signals that can recruit osteoclasts and osteoblasts[8] (Figure 1.2). Osteocyte-ablated mice are resistant to unloading-induced bone loss, providing evidence for the role of osteocytes in mechanotransduction[27].

Important signaling molecules produced by osteocytes in response to mechanical loading by a pulsating fluid flow are a.o. prostaglandins[28-30] and nitric oxide (NO)[30-37]. Osteopontin is rapidly upregulated by osteocytes after acute disuse, which may serve to mediate bone resorption, given that osteopontin acts as an osteoclast chemotaxant, and as a modulator of osteoclastic attachment to bone[38]. It has been shown that in cell culture experiments, osteocytes produce high levels of NO in response to fluid shear stress[30-37] and to a localized mechanical loading on the single osteocyte level[39,40]. NO is a short-lived highly reactive free radical involved in several biological processes, including the regulation of bone metabolism[36,41], and is produced through the activity of constitutive endothelial nitric oxide synthase (eNOS) and/or inducible nitric oxide synthase (iNOS)[41]. NO inhibits osteoclast activity[42], and mediates adaptive bone formation *in vivo*[43]. In endothelial cells, NO production in response to fluid flow plays a major role in preventing apoptosis[44].

During an orthodontic load osteoclasts accurately target their resorptive activity at the "pressure side" while osteoblasts are formed and activated at the "tension side" (Figure 1.1). What then determines the activity of the osteoblasts, and steers the resorption of the osteoclasts? It is generally assumed that the coordinated cooperation of osteoclasts and osteoblasts during bone adaptation is orchestrated by the osteocytes, which are able to respond to mechanical signals[8,13,45]. At the "pressure side" during orthodontic tooth movement where bone

resorption takes place, lowering of normal strain of the periodontal ligament occurs[46,47], which likely results in local stasis of extracellular fluid in the bone canalicular network. At the "tension side" during orthodontic tooth movement where bone deposition takes place, increased strain in bone caused by traction of the periodontal ligament results in increased fluid flow in the canaliculi[48]. The osteocytes that form a network throughout the strained matrix are thought to respond to these opposite flow patterns by facilitating osteoclastic activity at the "pressure side", where flow is low, and by stimulating osteoblastic bone formation around the "tension side", where flow is high. Osteoclast and osteoblast activity could thus be related to opposite flow patterns at the microscopical level, while the mechanosensitive osteocytes are of crucial importance for orchestrating the remodeling process[10].

Furthermore, osteocyte apoptosis has emerged as an important regulator of bone remodeling[9,49-52]. Studies in growing bone report that osteocyte apoptosis is associated with osteoclastic resorption[51,52]. Estrogen withdrawal in rats resulted in an increase in osteocyte apoptosis[53], suggesting that the loss of estrogen during the menopause alters the response to mechanical loading through compromising the viability of the osteocyte, thereby contributing to the rapid loss of bone during the menopause. The local mechanisms initiating osteocyte apoptosis are currently unknown, as well as the local signals that are produced by apoptotic osteocytes to attract or target osteoclasts. Nevertheless, the strong association between osteocyte apoptosis and subsequent bone remodeling supports the hypothesis that during bone remodeling, osteocyte apoptosis is the signal for continued osteoclastic bone resorption.

Objectives of the thesis

This thesis examines the role of mechanical loading in various aspects of bone adaptation such as occurs during an orthodontic tooth movement, at the cellular level. We hypothesize that the osteocytes play a key role in the adaptation of bone to mechanical loading, and that this bone adaptation process is at least partially regulated by NO. To test this hypothesis, we addressed the following scientific questions:

1. Is there a relationship between osteocyte density, bone remodeling parameters, gender, and osteoporosis?
2. Does mechanical stimulation by fluid flow inhibit tumor necrosis factor-α-induced apoptosis of osteocytes, and is the inhibition of osteocyte apoptosis mediated by loading-induced NO production?
3. Which apoptosis-related genes alter their expression in response to a physiological mechanical load by pulsating fluid flow in osteocytes, and is the alteration of apoptosis-related genes mediated by loading-induced NO production?
4. Are mechanically-stimulated osteocytes capable to modulate osteoclast formation and bone resorption via soluble factors such as NO?
5. Does orthodontic force stimulate constitutive eNOS and/or iNOS in rat osteocytes?

The aim of this thesis is to contribute to a better understanding of the biological processes involved in bone mechanotransduction by osteocytes, which will lead to more insight in the cell biological processes after orthodontic load. In the following section these questions are briefly explained and an overview of the thesis is given.

Overview of the thesis

Osteocytes act in close cooperation with osteoblasts during their incorporation in the bone matrix[54], and osteocyte density is suggested to reflect the result of the bone remodeling process[55]. Hence, increased knowledge of osteocyte density will likely contribute to a better understanding of bone biology, and we investigated the relationship between osteocyte density, bone remodeling parameters, gender, and osteoporosis (Chapter 2).

The importance of apoptosis, or programmed cell death, for normal tissue development and turnover in biology is increasingly recognized. During bone adaptation, local disuse of bone likely leads to a local lack of canalicular fluid flow and, therefore to a lack of fluid shear stress. This lack of fluid shear stress induces osteocyte apoptosis[49], which could be the cellular signal for osteoclast recruitment resulting in bone

resorption[9]. Osteoclastic attack is directed towards apoptotic osteocytes[51,52], suggesting a key regulatory role for osteocyte apoptosis in bone adaptation such as occurs after an orthodontic load. Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine which induces apoptosis[56], and elevated TNF-α levels are found in the gingival sulcus during orthodontic tooth movement[57]. We investigated if mechanical stimulation by fluid flow could inhibit TNF-α-induced apoptosis in osteocytes, and if the inhibition of osteocyte apoptosis is mediated by loading-induced NO production (Chapter 3).

Which apoptosis-related genes are altered in their expression in response to a physiological mechanical load in osteocytes is unknown. We studied apoptosis-related gene expression in response to mechanical loading by pulsating fluid flow in osteocytes, and we tested whether changes in gene expression were mediated by loading-induced NO production (Chapter 4).

Pericellular fluid may be critical in transmitting soluble mediators from osteocytes to other bone cells to produce a desired response to mechanical stimulation. In depth examination of the effects of conditioned medium from mechanically-stimulated osteocytes on osteoclast formation and activity can help to expand our understanding of the soluble factors released by osteocytes into the pericellular fluid. We investigated if mechanically-stimulated osteocytes are capable to modulate osteoclast formation and bone resorption via soluble factors such as NO (Chapter 5).

NO is produced by osteocytes through the activity of constitutive eNOS or iNOS. We hypothesized that eNOS and iNOS expression in osteocytes changes locally in alveolar bone in response to orthodontic force. We investigated eNOS and iNOS expression in osteocytes during orthodontic force application in a rat model (Chapter 6).

Finally, we discuss the relation between mechanotransduction by osteocytes and bone adaptation in the light of the experimental findings of this thesis (Chapter 7).

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CHAPTER 2

DIFFERENCES IN OSTEOCYTE DENSITY AND BONE HISTOMORPHOMETRY BETWEEN MEN AND WOMEN AND BETWEEN HEALTHY AND OSTEOPOROTIC SUBJECTS

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ABSTRACT

Bone defects related to osteoporosis develop with increasing age, and differ between males and females. It is currently thought that the bone remodeling process is supervised by osteocytes in a strain-dependent manner. We have shown an altered response of osteocytes from osteoporotic patients to mechanical loading, and osteocyte density is reduced in osteoporotic patients, which might relate to imperfect bone remodeling, leading to lack of bone mass and strength. Hence, information on osteocyte density will contribute to a better understanding of bone biology in males and females and to the assessment of osteoporosis. Osteocyte density as well as conventional histomorphometric parameters of trabecular bone were determined in cancellous iliac crest bone of healthy postmenopausal women and men and of osteoporotic women and men.

Osteocyte density was higher in healthy females than in healthy males and lower in osteoporotic females than in healthy females. Bone mass was reduced in osteoporotic patients, both male and female. In females, trabecular number was reduced, whereas in males, trabecular thickness was reduced and eroded surface was increased. There were no correlations between the parameter groups bone architecture, bone formation, bone resorption, and osteocyte density.

These results are consistent with impaired osteoblast function in osteoporotic patients and with a different mechanism of bone loss between men and women, in which osteocyte density might play a role. The reduced osteocyte numbers in female osteoporotic patients might relate to imperfect bone remodeling leading to lack of bone mass and strength.

Key words: osteocyte, osteoporosis, gender, cancellous bone, histomorphometry

INTRODUCTION

Osteocytes are mechanosensitive bone cells[1,2] that are thought to activate other bone cells to initiate remodeling activity in response to mechanical stimuli[3-9], thus playing a key role in the regulation of bone remodeling[7,10]. The coupling between osteoclast and osteoblast activity has been suggested to be mechanically regulated[11]. Bone cells from osteoporotic patients are impaired in their response to mechanical stress, suggesting that mechanical signals are poorly sensed in osteoporosis[12], leading to bone loss as also occurs under disuse conditions[13]. The quality of the bone architecture is reduced in osteoporotic patients as well[14,15]. The mechanism of bone loss has been reported to differ between men and women[16-19]. Women lose bone mainly by the loss of whole trabeculae[16-18], while in men, the trabecular architecture remains intact, but trabecular thickness is reduced[19]. Postmenopausal women show accelerated bone loss compared to men of similar age, leading to an increased risk of bone fracture or osteoporosis. Accelerated bone loss in women during menopause is associated with reduced levels of estrogen. Estrogen receptors are abundantly expressed in osteocytes[20], but the expression is less in other cells of the osteoblast lineage[21], suggesting that osteocytes are likely involved in the regulation of estrogen-mediated bone remodeling.

Osteocytes act in close cooperation with osteoblasts during their incorporation in the bone matrix[22,23]. Osteocyte density is suggested to reflect the result of the bone remodeling process[24,25], suggesting differences in osteocyte density between healthy and osteoporotic subjects, and males and females. In trabecular iliac crest bone, osteocyte density decreases with increasing age and is different between healthy and osteoporotic subjects[24,26]. Hence, increased knowledge of osteocyte density will likely contribute to a better understanding of bone biology in males and females and to the assessment of osteoporosis.

In this study, we investigated the relationship between osteocyte density, bone remodeling parameters, gender, and osteoporosis. The number and size of osteocytes as well as conventional

histomorphometric parameters of trabecular bone were determined in cancellous iliac crest bone of healthy postmenopausal women and men of similar age and in women and men with vertebral fractures.

MATERIALS AND METHODS

Subjects

Four groups of subjects were compared: male osteoporotic, female osteoporotic, male control, and female control. The male osteoporotic group consisted of 15 subjects (age 60.0 ± 11.0 years, mean ± SD) and the female osteoporotic group of 40 postmenopausal subjects (age 69.6 ± 11.1 years, mean ± SD), with untreated osteoporosis. Osteoporotic subjects had at least one spontaneous vertebral crush fracture unrelated to any intercurrent pathologic condition and leading to the diagnosis of primitive osteoporosis. A vertebral fracture was defined as a reduction of 20% or more in the anterior, middle, or posterior height of a previously unfractured or fractured vertebra. Patients using drugs with known effects on bone metabolism, alcohol abusers, and heavy smokers were excluded.

The male control group consisted of 21 subjects (age 67.3 ± 5.6 years, mean ± SD), and the female control group consisted of 13 postmenopausal women (age 64.4 ± 5.4 years, mean ± SD). Control subjects were healthy volunteers, who gave informed consent for the tests performed. Healthy control subjects were not taking any known medication to affect bone metabolism, and alcohol abusers and heavy smokers were excluded.

The ethical review boards of the respective universities approved the protocols, and all subjects gave informed consent.

Bone biopsies

All female and male control subjects and 35 female and 11 male osteoporotic subjects received two demethylchlortetracycline labels separated by an interval of 12 days. Transiliac bone biopsies were obtained from all subjects (2 cm posterior and inferior to the anterior-

superior iliac spine), using Bordier's trephine (8 mm inner diameter). All biopsies were taken within a period of 1 – 4 years prior to histological measurements. Biopsies were processed according to the methods described by Chappard *et al*.[27] directly after they were obtained. Undecalcified histological sections of 7 µm thickness were made with a Jung-K microtome. Four unstained sections were used for the measurement of fluorescent labels. Structural indices and osteocyte density were determined in non-serial sections, stained with Mallory's (eight sections) or modified Goldner's trichrome method[27] (eight sections) for light microscopy. Osteoclast surface was determined in six sections from 31 control and 30 osteoporotic subjects. Sections were stained for osteoclastic tartrate-resistant acid phosphatase (TRAP) and counterstained with phosphomolybdic aniline blue[28]. The storage time of the sections was similar for the control and osteoporotic groups. No deterioration in quality of the sections was observed with storage time.

Bone histomorphometry

Trabecular bone volume (BV/TV) and cancellous bone surface (BS) were measured with an automatic image analyzer (TAS+; Leitz, Wetzlar, Germany) at x25 magnification. Using these parameters, the architectural parameter wall thickness (W.Th) was calculated in structural units from the distance between cement lines and BS, which was quiescent and interfaced with the marrow space[29]. Parameters associated with bone formation were also measured. The distance between double fluorescent labels was used to calculate the mineral apposition rate (MAR). Furthermore, relative osteoid volume (OV/BV), osteoid surface (OS/BS), and osteoid thickness (O.Th) were measured. Parameters associated with bone resorption were the relative eroded surface (ES/BS) and the trabecular surface covered by osteoclasts (Oc.S/BS). The number of osteocytes per bone area (N.Ot/BAr) and the area of osteocyte lacunae (Lac.Ar) were assessed in trabecular bone. Osteocytes were stained red and easily visible in the bone matrix. Osteocyte number was assessed in 20 microscopic fields from two sections per subject by moving the sections in equally sized steps along the *x* and *y* axis. This way, a total BAr of approximately 1 mm^2 per

subject was measured. It should be noted that the number of osteocytes measured depends on the section thickness[26]. Therefore, the absolute values measured can vary between studies. Lac.Ar was measured in randomly selected fields using an oil immersion objective of x100 magnification. Each lacuna was outlined with a XY-tablet connected to a computer, and Lac.Ar was calculated from the digital measurement. At least 75 lacunae were outlined per trabecular BS in randomly selected fields, totaling at least 150 lacunae per subject[26]. For N.Ot and Lac.Ar, the intraobserver reproducibility was determined by measuring the parameters six times in four different subjects. From N.Ot and Lac.Ar, the total Lac.Ar/BAr was calculated using the equation Lac.Ar/BAr = [100 x (N.Ot/BAr) x Lac.Ar]/1000^2.

The histomorphometrical parameters were measured with a semiautomatic system (microscope and digitizing XY-tablet connected to a computer) at x100 magnification, except for MAR, W.Th, O.Th, Oc.S/BS, and Lac.Ar, where x250 magnification was used. The constant $\pi/4$ was used to convert two-dimensional values into three-dimensional values.

Statistics

Coefficients of variation were calculated for repeated measurements to assess the reproducibility of the N.Ot and Lac.Ar measurements. Comparison of means between groups was performed with nonparametric statistics, using the Kruskal-Wallis test. Linear regression and correlation coefficients between parameters were calculated using the method of least squares.

RESULTS

Bone architecture parameters revealed significant differences between healthy control subjects and osteoporotic patients (Table 2.1). Osteoporotic patients had significantly lower BV than healthy subjects. In osteoporotic women, the reduced BV was associated with a significantly reduced trabecular number and increased trabecular

Table 2.1 *Results of histomorphometric measurements for all groups and statistic comparison (Kruskal-Wallis Test) between groups.*

	Healthy subjects		Fracture patients		Healthy vs. patients		Females vs. males	
Variable (mean ± SD)	Females (n=13)	Males (n=21)	Females (n=40)	Males (n=15)	Females	Males	Healthy subjects	Fracture patients
Age (years)	64.4 ± 5.4	67.3 ± 5.6	69.6 ± 11.1	60.0 ± 11.0				
Bone architecture								
BV/TV (%)	22.8 ± 5.9	19.7 ± 6.4	14.2 ± 6.2	12.7 ± 5.4	P < 0.001	P < 0.001	NS	NS
Tb.Th (µm)	138 ± 32	133 ± 28	117 ± 31	101 ± 20	NS	P < 0.001	NS	NS
Tb.Sp (µm)	479 ± 105	581 + 182	790 ± 298	786 ± 318	P < 0.001	NS	P < 0.05	NS
Tb.N (mm^{-1})	1.66 ± 0.23	1.47 ± 0.30	1.19 ± 0.31	1.26 ± 0.41	P < 0.001	NS	NS	NS
W.Th (µm)	40.2 ± 5.5 (n=10)	48.4 ± 5.6 (n=20)	35.1 ± 5.5	34.1 ± 5.2	P < 0.05	P < 0.001	P < 0.01	NS
Bone formation								
OV/BV (%)	2.80 ± 1.75	2.93 ± 2.04	1.69 ± 1.21	1.97 ± 1.64	P < 0.01	NS	NS	NS
OS/BS (%)	14.0 ± 7.3	15.0 ± 7.9	8.8 ± 5.1	8.8 ± 5.4	P < 0.01	P < 0.05	NS	NS
O.Th (µm)	15.4 ± 4.0	11.7 ± 5.5	11.2 ± 4.9	8.1 ± 2.9	P < 0.01	P < 0.05	P < 0.05	P < 0.05
MAR (mm/day)	0.70 ± 0.08	0.66 ± 0.11	0.52 ± 0.21 (n=35)	0.63 ± 0.21 (n=11)	P < 0.05	NS	NS	NS
Bone resorption								
ES/BS (%)	4.37 ± 1.31	3.85 ± 1.38	5.69 ± 2.47	5.94 ± 2.33	NS	P < 0.01	NS	NS
Oc.S/BS (%)	2.53 ± 0.84 (n=12)	2.02 ± 0.89 (n=19)	1.42 ± 0.76 (n=20)	1.36 ± 1.29 (n=10)	P < 0.01	P < 0.05	NS	NS
Osteocytes								
N.Ot/BAr (mm^{-2})	271.3± 28.2	223.2± 29.9	222.6 ± 55.7	198.9± 54.2	P < 0.01	NS	P<0.001	NS
Lac.Ar (μm^2)	44.8 ± 7.7	41.3 ± 5.2	42.1 ± 5.1	40.7 ± 5.2	NS	NS	NS	NS
Lac.Ar/BAr (%)	1.20 ± 0.18 (n=13)	0.91 ± 0.14 (n=21)	0.94 ± 0.29 (n=38)	0.81 ± 0.27 (n=11)	P < 0.01	NS	P<0.001	NS

n, number of subjects studied, except if indicated otherwise

Parameter	**Abbreviation**	**Unit**
Bone architecture		
Bone volume	BV/TV	%
Trabecular thickness	Tb.Th	µm
Trabecular separation	Tb.Sp	µm
Trabecular number	Tb.N	mm^{-1}
Wall thickness	W.Th	µm
Bone formation		
Relative osteoid volume	OV/BV	%
Relative osteoid surface	OS/BS	%
Osteoid thickness	O.Th	µm
Mineral apposition rate	MAR	mm/d
Bone resorption		
Eroded surface	ES/BS	%
Osteoclast surface	Oc.S/BS	%
Osteocytes		
Number of osteocytes per bone area	N.Ot/BAr	mm^{-2}
Lacunar area	Lac.Ar	μm^2
Total lacunar area per bone area	Lac.Ar/BAr	%

separation compared to healthy women. In osteoporotic men, the reduced BV was associated with reduced trabecular thickness relative to healthy men. In both osteoporotic men and women, W.Th was significantly reduced compared to healthy controls. In addition, W.Th was greater in healthy men compared to healthy women, while no difference was seen in osteoporotic men compared to osteoporotic women (Table 2.1). Trabecular separation was greater in healthy males compared to healthy females.

Bone formation parameters revealed significant differences between control subjects and osteoporotic patients (Table 2.1). All osteoid indices as well as MAR were significantly decreased in osteoporotic women compared to healthy women. In osteoporotic males, osteoid surface was reduced and osteoid seams were thinner than in healthy males. Furthermore, osteoid thickness was significantly less in men compared to women, in healthy as well as osteoporotic subjects.

The bone resorption parameter ES was significantly higher in osteoporotic men compared to healthy control men (Table 2.1). In both males and females, the Oc.S/BS was significantly less in osteoporotic subjects as compared to healthy controls (Table 2.1).

N.Ot and Lac.Ar could not be measured in six osteoporotic patients due to poor staining quality of the osteocytes. The intraobserver reproducibility of the parameters N.Ot and Lac.Ar was satisfactory; the coefficient of variation was 6.6% for N.Ot per bone area, and 8.4% for Lac.Ar. N.Ot per bone area and total Lac.Ar per bone area were significantly reduced in osteoporotic women as compared to healthy women. Both N.Ot and total Lac.Ar per bone area were increased in healthy women as compared to healthy men (Table 2.1).

There were no correlations between the parameter groups bone architecture, bone formation, bone resorption, and osteocytes.

DISCUSSION

In this study, histomorphometric parameters were compared between

postmenopausal women and men of similar age, and between osteoporotic patients and healthy controls. All control subjects were alive and volunteered to participate in this study. We determined the conventionally measured bone histomorphometric parameters, as well as the number and size of the osteocytes. As far as we know, this is the first study comparing osteocyte density between men and women from the same age group. We found a difference in N.Ot, and in total Lac.Ar per bone area between healthy postmenopausal female subjects and male subjects of the same age. Females had about 15% more osteocytes than males, which might be related to gender-dependent regulation of bone growth and remodeling by sex hormones[21,30] as estrogen receptors have been identified in osteocytes[20] and in osteoblasts[21], and differences in estrogen receptor expression have been found in female and male bones[20]. Combined estrogen administration and mechanical loading synergistically affected bone formation in ulnae from female, but not male, rats[31-33]. Vashishth *et al*.[34] did not find gender-related differences in osteocyte lacunar density; however, cortical bone from patients with an age range from 16-73 years was studied. Jordan *et al*.[35] measured osteocyte density in trabecular bone from the femoral neck. Only few men were included in this study. Although they did not find significant differences between men and women, the values for osteocyte density were higher in women compared to men[35]. Healthy and osteoporotic subjects did not reveal differences in Lac.Ar, which was also observed by McCreadie *et al*.[36], suggesting that Lac.Ar does not play a role in osteoporotic fracture.

We found that osteoporotic bone contains less osteocytes than healthy bone, which was also observed by Qui *et al*.[24] but is in contrast with our previous findings[26] and findings from Jordan *et al*.[35]. Jordan *et al*.[35] found higher osteocyte density in hip fracture patients compared to post mortem patients. They looked at trabecular bone from the femoral neck; hence, their material is not directly comparable to the material we used and differences in osteocyte density may be site-specific. In our previous study and in the study of Jordan *et al*.[35], bone from autopsy patients was used, whereas in the present study bone from living healthy volunteers was used as control. According to Recker *et al*.[17], it is

questionable whether autopsy bone can be regarded as normal. It is unclear what the exact background is of the autopsy subjects; therefore, it is possible that patients were included with some form of secondary bone disease[17]. All control subjects in our study were healthy and fully active without any disease. Furthermore, the discrepancy between the current and our previous study may be partly explained by a confounding effect of the mixing of sexes since in our previous study the groups were not matched for gender.

Osteocyte deficiency could contribute to bone fragility, either by impairing the detection of fatigue microdamage or by reducing canalicular flow[24,37]. The number of osteocytes in bone reflects the number of osteoblasts embedded in the bone matrix since there is no evidence for osteocyte death in iliac cancellous bone[26,38,39]. A reduction in the number of osteocytes in osteoporotic bone may therefore be related to a decreased number of osteoblasts available for embedding in the matrix; it has been suggested that defective osteoblast recruitment and decreased maturation of pre-osteoblasts into osteoblasts are major factors contributing to bone loss in aging and osteoporosis[40,41].

In the present study, using human bone, N.Ot and osteocyte Lac.Ar were not significantly correlated to parameters of bone formation. In fetal rat bone, however, osteocyte density is increased by insulin-like growth factor I (IGF-I), which is associated with enhanced osteoblast activity and bone formation, and IGF-1 mRNA expression was strongly expressed in osteocytes after mechanical loading[42]. In addition, mechanical loading of rat bone affected both bone formation and osteocyte density[43].

Since it is difficult to obtain bone biopsies from healthy subjects, most studies with conventional histomorphometry use autopsy material. In the present study, bone from living healthy volunteers with no history of any known bone disease was used as control. Nevertheless, the trends in architectural parameters measured in healthy and osteoporotic subjects are consistent with previous reports using autopsy material[16,17,44,45]. Our findings confirm earlier observations that bone loss is primarily due to the loss of whole trabeculae in osteoporotic women, while trabecular thickness is reduced in osteoporotic men[14,15,46].

W.Th was greater in healthy men compared to women, which is in agreement with findings that W.Th decreases during aging in women but not in men[47]. The reduced values of W.Th in osteoporotic men and women, as well as bone formation parameters, indicate that bone formation is impaired in osteoporotic patients. The reduced osteoid indices and MARs in osteoporotic patients confirms findings by others[45].

The ES was increased in osteoporotic patients, indicating that overall turnover is reduced in these patients. The increased ES suggests that the reversal phase between bone resorption and bone formation is prolonged in osteoporotic patients.

Osteocytes are the most abundant cell type in bone and are well suited to sense mechanical signals; at present, there is evidence that osteocytes are involved in the mechanical regulation of bone turnover[48]. Our results demonstrate that the number of osteocytes embedded in the bone matrix depends on gender and differs between healthy and osteoporotic subjects. Osteoporotic patients showed reduced bone turnover and changed bone architecture, which is characteristic for osteoporosis and the results are consistent with impaired osteoblast function in osteoporotic patients. Also osteocyte numbers differ between osteoporotic and healthy subjects. This may present evidence that alterations occur within the whole population of cells of the osteoblastic lineage. No correlation was observed between osteocyte density and bone remodeling parameters. Further research is required to elucidate the interplay between the role of osteocytes, mechanical load, hormones, and other factors.

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CHAPTER 3

FLUID SHEAR STRESS INHIBITS TNF-α-INDUCED OSTEOCYTE APOPTOSIS

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ABSTRACT

Bone tissue can adapt to orthodontic load. Mechanosensing in bone is primarily a task for the osteocytes, which translate the canalicular flow resulting from bone loading into osteoclast and osteoblast recruiting signals. Apoptotic osteocytes attract osteoclasts, and inhibition of osteocyte apoptosis can therefore affect bone remodeling. Since TNF-α is a pro-inflammatory cytokine with apoptotic potency, and elevated levels are found in the gingival sulcus during orthodontic tooth movement, we investigated if mechanical loading by pulsating fluid flow affects TNF-α-induced apoptosis in chicken osteocytes, osteoblasts, and periosteal fibroblasts.

During fluid stasis, TNF-α increased apoptosis by more than two-fold in both osteocytes and osteoblasts, but not in periosteal fibroblasts. One-hour pulsating fluid flow (0.70 ± 0.30 Pa, 5 Hz) inhibited (-25%) TNF-α-induced apoptosis in osteocytes, but not in osteoblasts or periosteal fibroblasts, suggesting a key regulatory role for osteocyte apoptosis in bone remodeling after application of an orthodontic load.

Key words: osteocyte, TNF-α, mechanical loading, apoptosis, orthodontic tooth movement

INTRODUCTION

Mechanical adaptation of bone is a cellular process that allows bone to adjust its mass and structure to its mechanical environment[1,2]. Such remodeling occurs during orthodontic tooth movement, where osteoclast recruitment is needed for bone resorption on the pressure side, and osteoblast recruitment for bone formation on the tension side[3]. The recruitment and activation of osteoclasts and osteoblasts is related to the strain distribution during bone remodeling[4]. These mechanical changes are detected by osteocytes, the most abundant cell type in bone[1].

Osteocytes are derived from osteoblasts that have stopped producing bone matrix, and are literally buried in bone matrix. They are in contact with neighboring osteocytes via long cell processes, located in canaliculi, which are filled with interstitial fluid[5]. This three-dimensional network of interconnected cells is present throughout bone, and it is via this network that the osteocytes are positioned to regulate bone remodeling[1,6]. When bone is loaded, interstitial fluid is squeezed through the three-dimensional network, resulting in fluid flow. This flow results in a strain-driven movement of interstitial fluid, through the canaliculi and along the osteocyte processes, which is sensed and transduced by osteocytes[1,7].

Pulsating fluid flow provokes an immediate nitric oxide (NO) response in osteocytes *in vitro*[8]. NO inhibits osteoclast activity[9], and mediates adaptive bone formation *in vivo*[10]. If bone is unloaded, no flow through the canaliculi will occur, resulting in reduced osteocyte fluid shear stress stimulation, as well as NO production[8]. In endothelial cells, NO production in response to fluid flow prevents apoptosis[11]. Since osteocytes and endothelial cells respond similarly to mechanical stimulation by fluid shear stress with an upregulation of endothelial cell nitric oxide synthase (ecNOS) and increased NO production[6], we suggest that insufficient NO production, due to insufficient fluid flow, might cause apoptosis in osteocytes[12].

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine, and induces apoptosis in, among others, fibroblasts and osteoblasts[13,14].

In bone, it stimulates osteoclastogenesis and inhibits osteoblast function[15], and elevated TNF-α levels are found in osteoporosis, periodontitis[16,17], and in the gingival sulcus during orthodontic tooth movement[18]. On the pressure side, lowering of normal strain from the functioning periodontal ligament occurs[19], which might result in local stasis of extracellular fluid in the canalicular network[4,20], a lack of fluid shear stress on the osteocytes, and reduced NO production. Fluid stasis in combination with TNF-α then induces osteocyte apoptosis on the pressure side.

Osteocyte apoptosis could be the signal for osteoclast recruitment to resorb bone on the pressure side, enabling the teeth to move[2]. Osteoclastic attack is directed towards apoptotic osteocytes[21,22], suggesting a key regulatory role for osteocyte apoptosis in bone remodeling, such as occurs after orthodontic load application.

Here, we studied osteocyte apoptosis induced by TNF-α after pulsating fluid flow (PFF) application. PFF, resulting in fluid shear stress on the cells, mimicked the manner in which loading of whole bones is thought to be conveyed to osteocytes *in vivo*[1,7]. Static cultures represented disuse conditions. Apoptosis was assessed as caspase-3/7 activity, since this enzyme mediates TNF-α-induced apoptosis[23]. The response of TNF-α-treated bone cells to mechanical loading was studied by measuring NO production. To validate that NO may modulate apoptosis, the release of NO was inhibited by N^G-Nitro-L-Arginine Methyl Ester (L-NAME), and apoptosis was assessed. We hypothesized that mechanical stimulation by fluid flow inhibits TNF-α-induced apoptosis in osteocytes, but not in osteoblasts or periosteal fibroblasts.

MATERIALS AND METHODS

Isolation and culture of osteocytes, osteoblasts, and periosteal fibroblasts

The use of fetal chicken calvariae satisfied the requirements of the Animal Ethical Committee of the Vrije Universiteit. Fetal chicken calvarial cells were isolated as described previously[5]. Osteocytes were separated from osteoblasts by immunomagnetic separation[24].

Osteocytes, osteoblasts, and periosteal fibroblasts were seeded at 7500 cells *per* spot (diameter 0.8 cm), with 2 spots *per* polylysine-coated (50 µg/ml; poly-L-lysine hydrobromide, mol wt 15-30 x 10^4; Sigma, St. Louis, MO, USA) glass slide, and left to attach overnight in alpha minimum essential medium (α-MEM; Gibco, Paisley, Scotland) supplemented with 2% chicken serum (Gibco), 200 µg/ml glutamine (Sigma), 50 µg/ml gentamycin sulfate (Sigma), 50 µg/ml L-ascorbic acid (Merck, Darmstadt, Germany), and 1 mg/ml D-glucose (Merck, Darmstadt, Germany) at 37°C in a humidified atmosphere of 5% CO_2 in air. After induction of apoptosis, cells were subjected to PFF.

Apoptosis

TNF-α (Imgen Technologies, Alexandria, VA, USA) at 5, 10, 25, 50, and 100 ng/ml, was added to osteoblasts, seeded at 3000 cells/well of a 96-well plate. Cells were incubated for 6, 16, and 24 hrs with TNF-α-containing serum-free α-MEM with 1% bovine serum albumin.

Pulsating fluid flow (PFF)

After induction of apoptosis with 10 ng/ml TNF-α for 16 hrs, osteocytes, osteoblasts, and periosteal fibroblasts were either or not subjected to 1 hr of PFF through a parallel-plate flow chamber containing the cells as described previously[6]. The cells were subjected to a 5 Hz pulse with a mean shear stress of 0.7 Pa, a pulse amplitude of 0.3 Pa, and a peak shear stress rate of 8.4 Pa/sec. Static cultures were kept in a petridish under conditions similar to those for the experimental cultures. After 10 and 30 min of PFF treatment or static culture, medium was collected and assayed for NO production. After 1 hr of PFF treatment or static culture, fresh medium was added, and cells were post-incubated under static conditions for 24 hrs.

Caspase-3/7 activity and DNA content

We created a culture well (diameter 1.5 cm) around the cell spot by securing a silicone rubber incubation ring onto the glass slide. A cell-lysis-based reagent of the Caspase-Glo 3/7 Assay (Promega, Madison,

WI, USA) was added for the assessment of caspase-3/7 activity with a luminometer (Berthold Technologies, Bad Wildbad, Germany), according to the manufacturer's instructions. DNA in the cell lysate was determined by a CyQUANT Cell Proliferation Assay (Molecular Probes Inc., Eugene, OR, USA). Cell apoptosis was expressed in relative light units *per* ng DNA.

Nitric oxide

NO was measured as nitrite accumulation in the medium, with Griess reagent[25] consisting of 1% sulfanilamide, 0.1% naphtylethylene-diamine-dihydrochloride, and 2.5 M H_3PO_4. Serial dilutions of $NaNO_2$ in medium were used for a standard curve. Absorbance was measured at 540 nm.

Inhibition of nitric oxide production

NO release was inhibited by the addition of 1 mM L-NAME (Sigma). The NO inhibitor was added to the culture medium during exposure to PFF.

Statistics

Treatment-over-control ratios and NO production data were analyzed by a paired *t*-test. Differences were considered significant when $p < 0.05$.

RESULTS

To determine maximal apoptotic induction by TNF-α, we added various concentrations (from 5 to 100 ng/ml) to osteoblasts for 6, 16 and 24 hrs. These dose-response and time-course studies showed that maximal induction of apoptosis was achieved by 10 ng/ml TNF-α after 16 hrs of treatment (Figure 3.1 A, B). Lower and higher concentrations of TNF-α for 16 hrs resulted in less induction of apoptosis (Figure 3.1 B), and 16 hrs was the earliest time-point at which apoptosis could be detected (Figure 3.1 A). Similar results were obtained for osteocytes (data not shown). Therefore 10 ng/ml TNF-α for 16 hrs was used to induce apoptosis for fluid flow experiments.

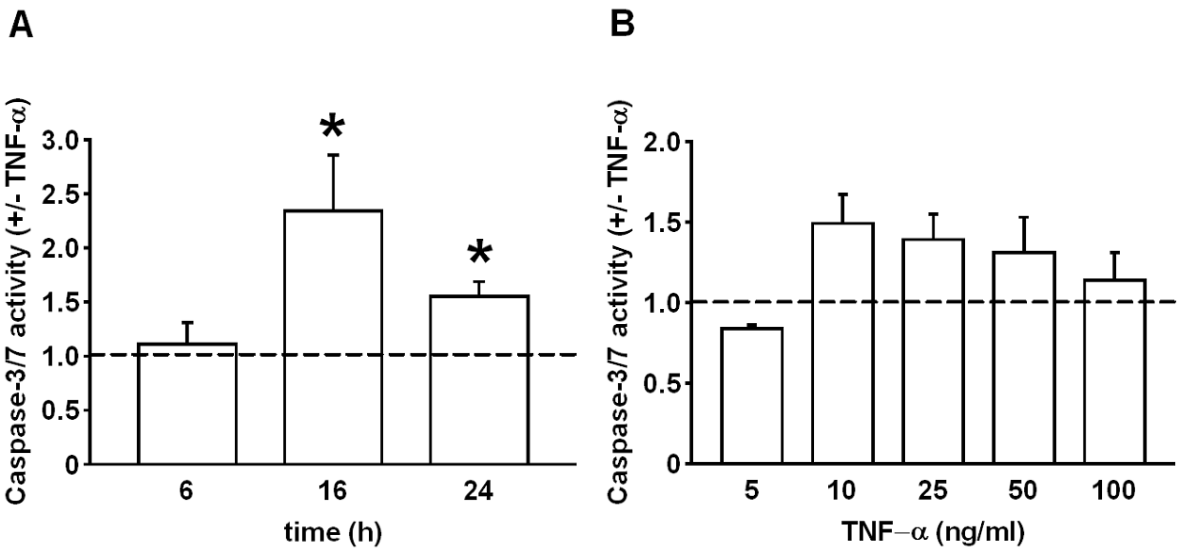

Figure 3.1 *Effects of TNF-α-induction time (A) and TNF-α-dose (B) on caspase-3/7 activity in osteoblasts under static conditions. (A) The earliest time point at which apoptosis could be detected was after 16 hrs of 10 ng/ml TNF-α treatment. (B) Maximal induction of apoptosis was achieved by 10 ng/ml TNF-α. Higher and lower concentrations of TNF-α resulted in less induction of apoptosis. Values, obtained from 3 wells of 1 experiment, are expressed as mean ± SEM of TNF-α-treated-over-control ratios (+/- TNF-α at 10 ng/ml [A]; +/- TNF-α at 5, 10, 25, 50, 100 ng/ml [B]). Dashed line, +/- TNF-α = 1 (no effect).*
** Significant effect of TNF-α, $p < 0.05$.*

TNF-α at 10 ng/ml for 16 hrs increased caspase-3/7 activity in osteocytes and osteoblasts by more than two-fold, compared with untreated cells (Figure 3.2). However, TNF-α did not affect the caspase-3/7 activity of periosteal fibroblasts (Figure 3.2).

After 16 hrs of apoptosis induction by 10 ng/ml TNF-α, one-hour PFF was applied to osteocytes, osteoblasts, and periosteal fibroblasts. No cells were removed by the application of PFF, as assessed by total DNA quantification (periosteal fibroblasts, static 21.4 ± 2.9 ng/ml, PFF 19.3 ± 2.0 ng/ml; osteoblasts, static 11.3 ± 1.2 ng/ml, PFF 10.4 ± 1.5 ng/ml; osteocytes, static 3.4 ± 1.0 ng/ml, PFF 4.2 ± 1.2 ng/ml; mean ± SEM of 6-8 experiments). Furthermore, PFF did not result in visible changes in cell shape or alignment in the direction of the flow.

One-hour PFF reduced caspase-3/7 activity in TNF-α-treated osteocytes by 25% compared with static cultures, at 24 hrs post-incubation without loading (Figure 3.3 A). However, PFF did not affect caspase-3/7 activity in osteoblasts or periosteal fibroblasts (Figure 3.3

A). Inhibition of apoptosis in osteocytes subjected to one-hour PFF was detectable at 24 hrs post-incubation without loading, but not immediately after PFF treatment (data not shown). To study a possible interrelationship between enhanced NO production resulting from PFF treatment and apoptosis, we subjected cells to PFF in the presence of L-NAME. The addition of 1 mM L-NAME to the culture medium during exposure to PFF blocked the inhibitory effect of PFF on caspase-3/7 activity in TNF-α-treated osteocytes, but did not affect apoptosis in osteoblasts and periosteal fibroblasts (Figure 3.3 B).

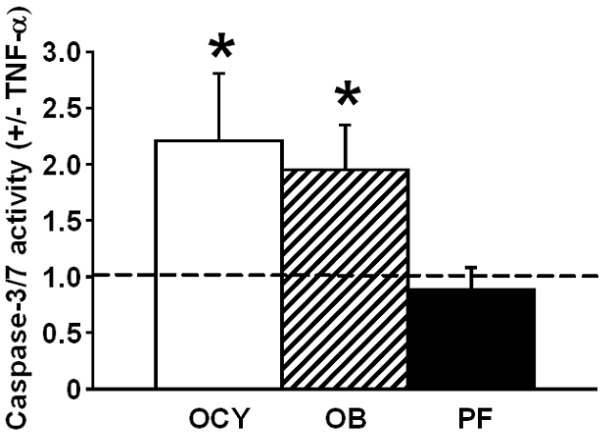

Figure 3.2 *Effect of TNF-α on apoptosis in osteocytes, osteoblasts, and periosteal fibroblasts under static conditions. TNF-α at 10 ng/ml for 16 hrs increased caspase-3/7 activity in osteocytes and osteoblasts by more than two-fold, but not in periosteal fibroblasts. Values, obtained from 4 (osteoblast and osteocyte) or 3 (periosteal fibroblast) separate experiments, are expressed as mean ± SEM of TNF-α-treated-over-control ratios (+/- TNF-α at 10 ng/ml). Dashed line, +/- TNF-α = 1 (no effect). OCY, osteocytes; OB, osteoblasts; PF, periosteal fibroblasts. * Significant effect of TNF-α, $p < 0.05$.*

Under static conditions, bone cells showed basal NO production, which was highest in TNF-α-treated osteocytes (Figure 3.4 A). PFF significantly increased NO production at 30 min, but not at 10 min in osteocytes (Figure 3.4 A). When data were expressed as PFF-treated-over-control ratios, the increase (4.8-fold) in TNF-α-treated osteocytes was even more clear (Figure 3.4 B). Such significant increase was not seen in osteoblasts (1.8-fold) or periosteal fibroblasts (2.0-fold) (Figure 3.4 B). Addition of 1 mM L-NAME during PFF inhibited PFF-mediated NO synthesis (data not shown), which was described previously[8].

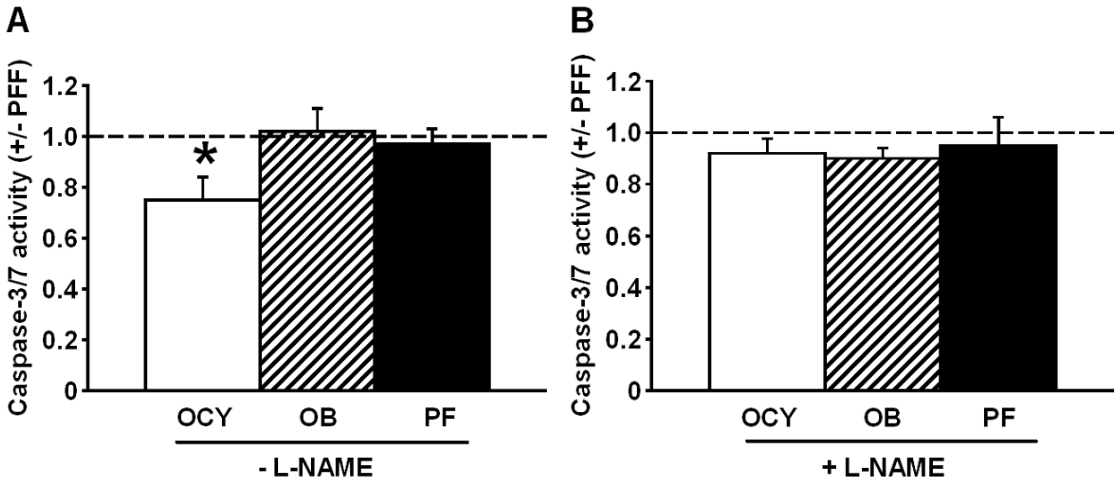

Figure 3.3 *Effect of pulsating fluid flow on TNF-α-induced apoptosis in osteocytes, osteoblasts, and periosteal fibroblasts. Apoptosis was induced by TNF-α at 10 ng/ml for 16 hrs. (A) Apoptosis in the absence of L-NAME. One hour of PFF reduced caspase-3/7 activity in TNF-α-treated osteocytes by 25%, but not in osteoblasts and periosteal fibroblasts. Values obtained from 6 osteoblast, 7 periosteal fibroblast, and 8 osteocyte experiments are expressed as mean ± SEM of PFF-treated-over-control ratios (+/- PFF). (B) Apoptosis in the presence of L-NAME. Addition of 1 mM L-NAME to the culture medium during exposure to PFF blocked the inhibitory effect of PFF on caspase-3/7 activity in TNF-α-treated osteocytes, but did not affect apoptosis in osteoblasts and periosteal fibroblasts. Values, obtained from 3 experiments, are expressed as mean ± SEM of PFF-treated-over-control ratios (+/- PFF). Dashed line, +/- PFF = 1 (no effect). PFF, pulsating fluid flow; OCY, osteocytes; OB, osteoblasts; PF, periosteal fibroblasts; L-NAME, N^G-Nitro-L-Arginine Methyl Ester. * Significant effect of PFF, $p < 0.05$.*

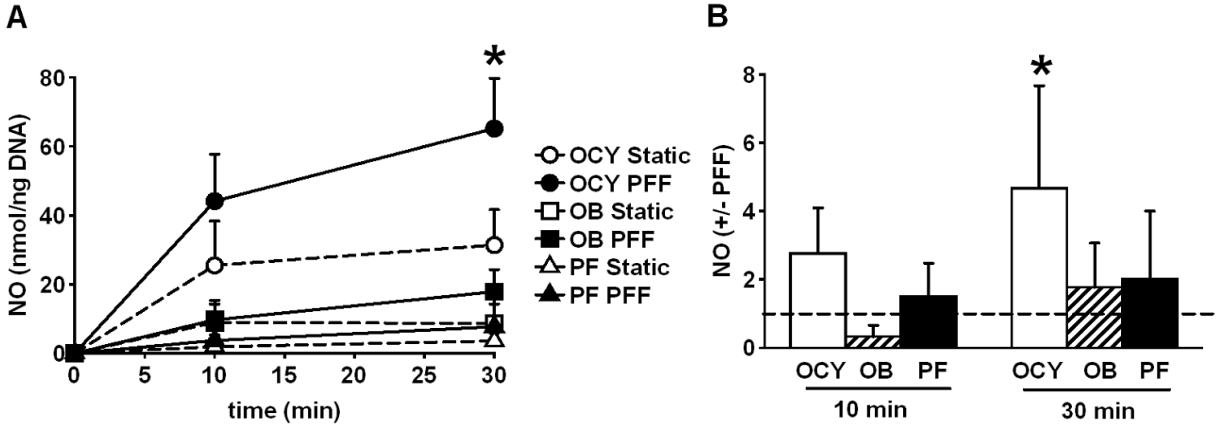

Figure 3.4 *Effect of pulsating fluid flow on NO production by TNF-α-treated osteocytes, osteoblasts, and periosteal fibroblasts. (A) Cumulative NO production during 30 min of PFF treatment or under static conditions. (B) NO production expressed as PFF-treated-over-control ratios (+/- PFF) at 10 and 30 min. Application of PFF increased NO production at 30 min in TNF-α-treated osteocytes, but not in osteoblasts and periosteal fibroblasts. Values are obtained from 4-5 glass slides from 2 experiments (mean ± SEM). Dashed line, +/- PFF = 1 (no effect). PFF, pulsating fluid flow; Stat, static cultures; OCY, osteocytes; OB, osteoblasts; PF, periosteal fibroblasts. *Significant effect of PFF, $p < 0.05$.*

DISCUSSION

We found that TNF-α increased apoptosis in osteocytes and osteoblasts, but not periosteal fibroblasts, under static conditions representing a situation of no fluid shear stress along the cell membrane. We are the first to show that mechanical loading by pulsating fluid flow of physiological magnitude reduces TNF-α-induced apoptosis in osteocytes, but not osteoblasts and periosteal fibroblasts. The reduction of apoptosis was not due to removal of pre-apoptotic osteocytes by shear stress, since there was no reduction of DNA content. Inhibition of NO synthesis by L-NAME prevented the PFF-mediated downregulation of apoptosis in osteocytes, suggesting that PFF-mediated NO release by osteocytes is, at least in part, responsible for the loading-induced inhibition of osteocyte apoptosis. We conclude that for terminally differentiated osteocytes, fluid shear stress seems to be an important survival factor, while no such sensitivity is expressed by the less mature osteoblasts and periosteal fibroblasts containing osteoprogenitors.

Others have described inhibition of MLO-Y4 cell apoptosis by stretching[26], and rat osteoblast apoptosis by steady fluid shear stress[13]. However, we have subjected our cells to a pulsating fluid shear stress regime, which mimics the manner by which loading of whole bones is thought to be conveyed to the osteocytes *in vivo*[1,7]. Earlier, we showed inhibition of serum-starvation-induced apoptosis by mechanical stimulation[12]. During inflammation and orthodontic tooth movement, however, the cytokine TNF-α is produced. We found that the inhibitory effect of PFF on TNF-α-induced osteocyte apoptosis was less pronounced then after serum starvation, suggesting that mechanical loading is less effective in inhibiting TNF-α-induced apoptosis.

TNF-α stimulates apoptosis of murine osteoblasts and osteocytic MLO-Y4 cells[27]. We observed that periosteal fibroblasts fail to undergo apoptosis in response to TNF-α at 10 ng/ml. Higher concentrations of TNF-α (20 ng/ml) stimulate apoptosis in human fibroblasts[14], suggesting that TNF-α affects apoptosis of mature bone cells, such as osteocytes and osteoblasts, but that periosteal fibroblasts are less sensitive.

The signal transduction pathway leading from fluid shear stress to

inhibition of osteocyte apoptosis is currently unknown, but it is likely that NO is involved. Osteocytes rapidly (within minutes) produce low amounts of NO in response to shear stress[8]. NO inhibits apoptosis in endothelial cells via inhibition of caspase-3[11]. Here we show that fluid shear stress also stimulates NO production in TNF-α-treated osteocytes, and that the inhibition of NO production by L-NAME prevented the PFF-mediated downregulation of apoptosis in osteocytes. This suggests that NO is a mediator of mechanical effects in bone, leading to inhibition of apoptosis.

Verification of our *in vitro* results is needed in human bone cells, and ultimately, in whole human bones, before definitive conclusions can be drawn, but a relation between stress-related osteocyte survival and bone remodeling seems likely. Local fatigue damage of the extracellular matrix results in loss of osteocyte integrity and activates bone remodeling[28,29]. Our results are well compatible with this concept if we consider that fatigue damage will lead to reduced canalicular fluid flow[30]. Reduced canalicular shear stress will promote osteocyte apoptosis, which will attract osteoclasts, thereby activating remodeling[21].

Our results might offer, at least in part, an explanation for the complex process of orthodontic tooth movement, in which many cells and cytokines are involved. Periodontal ligament cells are stretched or compressed[3], and cytokines such as TNF-α and IL-1 are produced[18]. We suggest that, together with TNF-α in the gingival sulcus, osteocyte apoptosis is caused on the pressure side by local stress shielding due to decreased functioning of the periodontal ligament[18,19,31], which causes almost complete fluid stasis in the canaliculi of the osteocytes[20]. Osteoclasts are then attracted by apoptotic osteocytes[21], resulting in bone resorption and remodeling. On the tension side, increased strain likely results in increased fluid flow, which stimulates osteocytes to produce NO, thereby maintaining osteocyte viability, and osteoblasts to produce new bone. *In vivo* observations by Hayashi *et al*.[32] showed inhibition of orthodontic tooth movement in rats treated with the NO inhibitor L-NAME. This inhibition might be explained by decreased bone formation on the tension side.

In summary, TNF-α induces apoptosis of mature bone cells, i.e., osteocytes and osteoblasts, but not immature bone cells of the periosteal fibroblast population. Fluid shear stress inhibits TNF-α-induced apoptosis specifically in osteocytes, but not osteoblasts and periosteal fibroblasts. This inhibitory effect is, at least in part, mediated by NO. This suggests a regulatory role for osteocyte apoptosis in osteoclastic bone resorption during bone remodeling, such as occurs after the application of an orthodontic load.

ACKNOWLEDGEMENTS

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CHAPTER 4

INHIBITION OF OSTEOCYTE APOPTOSIS BY FLUID FLOW IS MEDIATED BY NITRIC OXIDE

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ABSTRACT

Bone unloading results in osteocyte apoptosis, which attracts osteoclasts leading to bone loss. Loading of bone drives fluid flow over osteocytes which respond by releasing signaling molecules, like nitric oxide (NO), that inhibit osteocyte apoptosis and alter osteoblast and osteoclast activity thereby preventing bone loss. However, which apoptosis-related genes are modulated by loading is unknown. We studied apoptosis-related gene expression in response to pulsating fluid flow (PFF) in osteocytes, osteoblasts, and fibroblasts, and whether this is mediated by loading-induced NO production. PFF (0.7 ± 0.3 Pa, 5 Hz, 1 hr) upregulated Bcl-2 and downregulated caspase-3 expression in osteocytes. L-NAME attenuated this effect. In osteocytes PFF did not affect p53 and c-Jun, but L-NAME upregulated c-Jun expression. In osteoblasts and fibroblasts PFF upregulated c-Jun, but not Bcl-2, caspase-3, and p53 expression. This suggests that PFF inhibits osteocyte apoptosis via alterations in Bcl-2 and caspase-3 gene expression, which is at least partially regulated by NO.

Key words: osteocyte, mechanical loading, fluid flow, apoptosis, Bcl-2, caspase-3, c-Jun, p53, nitric oxide, orthodontic tooth movement

INTRODUCTION

Bone continuously remodels in such a way that bones in the adult skeleton show an optimal morphology to withstand the stresses placed upon them, with a minimal use of material[1,2]. Thus when bone is unloaded, bone formation, mineral content, and bone matrix protein production decrease. The opposite is also true, i.e. mechanical loading stimulates bone formation and increases bone mineral density *in vivo*[1,2]. This process of mechanical adaptation plays an important role during orthodontic tooth movement, where the recruitment and activation of osteoclasts and osteoblasts are related to the load distribution during orthodontic tooth movement[3]. On the pressure side, a decrease in normal strain occurs as a result of unloading of the periodontal ligament, which likely results in local unloading of bone followed by bone resorption[4]. On the tension side, the increased pulling force on the periodontal ligament is transferred to the bone. The resulting increase in bone loading on the tension side likely contributes to osteoblast recruitment and bone formation[4].

The process of mechanical adaptation of bone is governed by osteocytes, which are derived from osteoblasts that have stopped producing bone matrix, and are literally buried within the bone matrix[1]. The osteocyte cell bodies are positioned in lacunae, and they are in contact with neighboring osteocytes via long slender cell processes located in canaliculi, which are filled with interstitial fluid[5]. When bones are loaded, the resulting strain acts as a driving force which causes a flow of interstitial fluid through the lacuno-canalicular network[1]. This fluid flow is sensed by osteocytes[1,6,7], which respond by producing signaling molecules that stimulate osteoblast – and inhibit osteoclast recruitment and activity, resulting in a gain of bone mass[8,9]. During skeletal unloading, the lack of fluid shear stress leads to a diminished production of signaling molecules resulting in the stimulation of osteoclast activity and enhanced bone resorption[9]. It has been postulated that prolonged unloading leads to osteocyte apoptosis, which promotes osteoclastic bone resorption[2].

Nitric oxide (NO) is a short-lived, highly reactive free radical

involved in several biological processes, including the regulation of bone metabolism[10]. NO inhibits osteoclast activity[11] and mediates the anabolic response of bones to mechanical loading *in vivo*[12]. We and others have shown that in cell culture experiments, osteocytes produce high levels of NO in response to loading in the form of a physiologic fluid shear stress[13,14]. Loading-induced NO production in bone cells results from the activity of endothelial cell nitric oxide synthase (ecNOS)[14]. In endothelial cells, NO production by ecNOS in response to pulsating fluid flow (PFF) plays a major role in preventing apoptosis[15,16]. Osteocytes might thus likewise be protected against apoptosis by a basal amount of NO production under normal canalicular shear stress during physiological mechanical loading. Conversely, during unloading osteocytes might enter apoptosis as a result of insufficient NO production due to insufficient fluid flow in the canaliculi. Previously, we have shown that mechanical loading by PFF of physiological magnitude reduces TNF-α-induced apoptosis in osteocytes[17]. Inhibition of NO synthesis by N^G-Nitro-L-Arginine Methyl Ester (L-NAME) prevented the PFF-mediated downregulation of apoptosis in osteocytes, suggesting that NO release by osteocytes is, at least in part, responsible for loading-induced inhibition of osteocyte apoptosis[17].

The aim of this study was to elucidate which apoptosis-related genes alter their expression in response to a physiological mechanical load, and whether such an alteration is mediated by loading-induced NO production. To validate that NO may modulate apoptosis-related gene expression in osteocytes, we inhibited the release of NO by L-NAME. We have measured gene expression of Bcl-2, caspase-3, p53, and c-Jun, because these molecules are key-regulating molecules of apoptosis.

MATERIALS AND METHODS

Isolation and culture of osteocytes, osteoblasts, and periosteal fibroblasts

The use of fetal chicken calvariae satisfied the requirements of the Animal Ethical Committee of the Vrije Universiteit. Fetal chicken calvarial cells were isolated as described earlier[5]. Osteocytes were

separated from osteoblasts by immunomagnetic separation[18]. Osteocytes, osteoblasts, and periosteal fibroblasts were then seeded at 3 x 10^4 cells/cm^2 on polylysine-coated (50 mg/ml; poly-L-lysine hydrobromide, mol wt 15-30 x 10^4; Sigma, St. Louis, MO, USA) glass slides, and left to attach overnight in alpha minimum essential medium (Gibco, Paisley, Scotland) supplemented with 2% chicken serum (Gibco), 200 µg/ml glutamine (Sigma), 50 µg/ml gentamicin sulfate (Sigma), 50 µg/ml L-ascorbic acid (Merck, Darmstadt, Germany), and 1 mg/ml D-glucose (Merck) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Pulsating fluid flow (PFF)

Osteocytes, osteoblasts, and periosteal fibroblasts were either or not subjected to 1 hr of PFF through a parallel-plate flow chamber containing the cells as described previously[19]. The cells were subjected to a 5 Hz pulse with a mean fluid shear stress of 0.7 Pa, a pulse amplitude of 0.3 Pa, and a peak shear stress rate of 8.4 Pa/sec. Static cultures were kept in a petridish under similar conditions as the experimental cultures.

Nitric oxide

NO release was inhibited by adding 1 mM L-NAME (Sigma) to the culture medium during exposure of the cells to PFF. NO was measured as nitrite in the medium, using Griess reagent[20] consisting of 1% sulfanilamide, 0.1% naphtylethelene-diamine-dihydrochloride, and 2.5 M H_3PO_4. Serial dilutions of $NaNO_2$ in medium were used as standard curve. Absorbance was measured at 540 nm.

DNA content

DNA content was determined in osteocytes, osteoblasts, and periosteal fibroblasts after 1 hr of pulsating fluid flow or static conditions in the cell lysate using a CyQUANT Cell Proliferation Assay (Molecular Probes Inc., Eugene, OR, USA).

Analysis of gene expression

Total RNA was extracted from non-stressed control cultures and cells subjected to 1 hr PFF by using TRIzol Reagent (Gibco), according to the manufacturer's instructions. cDNA synthesis was performed using 0.1-0.75 µg total RNA in a 20 µl reaction mix consisting of 5 units Transcriptor Reverse Transcriptase (Roche Diagnostics, Basel, Switzerland), 0.08 A260 units random primers (Roche Diagnostics), 1 mM of each dNTP (Invitrogen, Carlsbad, CA, USA), and 1x concentrated Transcriptor RT reaction buffer. Real-time PCR reactions were performed using the LightCycler® 480 SYBR green I Master reaction mix according to the manufacturer's instructions in a LightCycler 480 (Roche Diagnostics), and mRNA expression for Bcl-2, caspase-3, p53, and c-Jun were determined, and were normalized for 18S gene expression. Primers (Invitrogen) used for real-time PCR are listed in Table 4.1.

Table 4.1 *Primers used for real time PCR*

Target gene	Forward primer	Reverse primer	Product size (bp)
18S	5'-gtaacccgttgaaccccatt-3'	5'-ccatccaatcggtagtagcg-3'	180
Bcl-2	5'-ggaaacttgacagaggatcat-3'	5'-atcacgcggaacacttgatt-3'	185
Caspase-3	5'-cagatgctgcaagtgtcagag-3'	5'-gtttcatctggtccactgtc-3'	337
p53	5'-atcggtcacctgcacttactc-3'	5'-agcaccgtggtacagtcaga-3'	342
c-Jun	5'-tgacatggagtcgcaggaga-3'	5'-ccgctgttgacatggttc-3'	222

Bcl-2, B-cell lymphoma 2.

Statistics

Student's *t*-test was used. Differences were considered significant when $p < 0.05$.

RESULTS

No cells were removed by application of PFF, as assessed by visual inspection of the cultures before and after treatment, and by total DNA quantification (periosteal fibroblasts, static 21.4 ± 2.9 ng/ml, PFF 19.3 ±

2.0 ng/ml; osteoblasts, static 11.3 ± 1.2 ng/ml, PFF 10.4 ± 1.5 ng/ml; osteocytes, static 3.4 ± 1.0 ng/ml, PFF 4.2 ± 1.2 ng/ml; mean ± SEM of 6-8 experiments). Furthermore, PFF did not result in visible changes in cell shape or alignment of the cells in the direction of the flow. Osteocytes exhibited long, branched cytoplasmic processes radiating from a relatively round cell body. Osteoblasts were more cuboidal and periosteal fibroblasts more spindle shaped.

Under static control conditions, osteocytes, osteoblasts, and periosteal fibroblasts showed basal NO production, which was highest in osteocytes (Figure 4.1). PFF significantly increased NO production at 30 min in osteocytes, but not in osteoblasts or periosteal fibroblasts. Addition of 1 mM L-NAME during PFF inhibited the PFF-mediated NO production in osteocytes (data not shown), which has been described earlier[13].

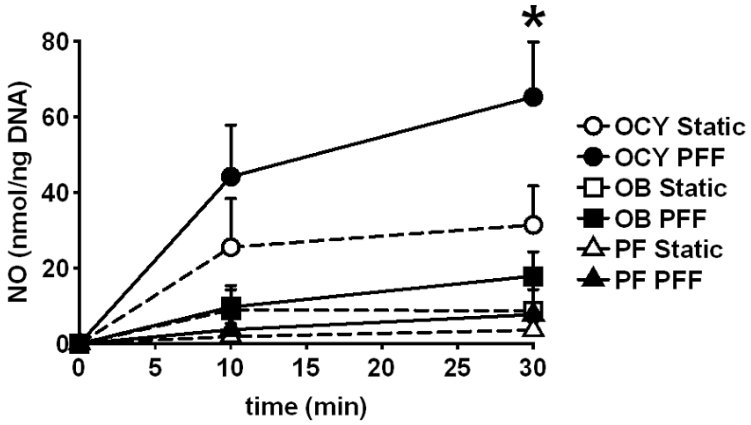

Figure 4.1 *Effect of pulsating fluid flow on NO production by osteocytes, osteoblasts, and periosteal fibroblasts. Cumulative NO production during 30 min of PFF treatment or under static conditions is shown. Application of PFF increased NO production at 30 min in osteocytes, but not in osteoblasts and periosteal fibroblasts. Values, obtained from 4-5 glass slides from two experiments, are expressed as mean ± SEM. PFF, pulsating fluid flow; Stat, static cultures; PF, periosteal fibroblasts; OB, osteoblasts; OCY, osteocytes. * Significant effect of PFF, $p < 0.05$.*

One-hour PFF upregulated Bcl-2 gene expression in osteocytes by 3-fold compared with static control cultures (Figure 4.2 A). PFF did not affect Bcl-2 gene expression in osteoblasts or periosteal fibroblasts (Figure 4.2 A). One-hour PFF downregulated caspase-3 gene expression

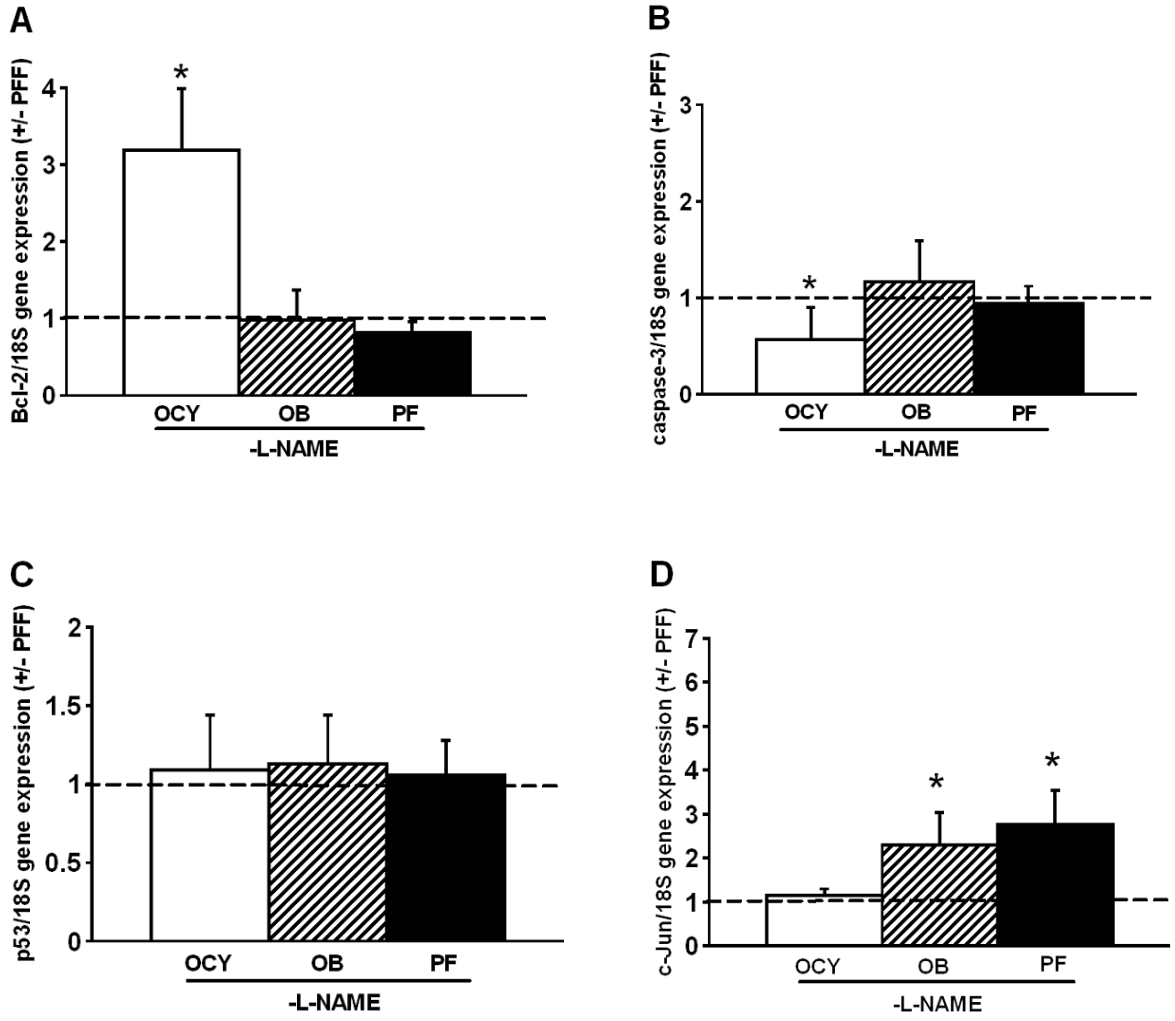

Figure 4.2 *Effect of pulsating fluid flow on Bcl-2, caspase-3, p53, and c-Jun gene expression in osteocytes, osteoblasts, and periosteal fibroblasts in the absence of L-NAME. (A) Bcl-2 gene expression in the absence of L-NAME. One hour of PFF upregulated Bcl-2 gene expression in osteocytes by more than 3-fold, but not in osteoblasts and periosteal fibroblasts. (B) caspase-3 gene expression in the absence of L-NAME. One hour of PFF downregulated caspase-3 gene expression in osteocytes by more than 40%, but not in osteoblasts and periosteal fibroblasts. (C) p53 gene expression in the absence of L-NAME. One hour of PFF did not affect p53 gene expression in osteocytes, osteoblasts, and periosteal fibroblasts. (D) c-Jun gene expression in the absence of L-NAME. One hour of PFF upregulated c-Jun gene expression in osteoblasts (2.3-fold) and periosteal fibroblasts (2.8-fold), but did not affect c-Jun gene expression in osteocytes. Values, obtained from 3-5 osteocyte, and 5-6 osteoblast and periosteal fibroblast experiments, are expressed as mean ± SEM of PFF-treated-over-control ratios (+/- PFF). Dashed line, +/- PFF = 1 (no effect). PFF, pulsating fluid flow; PF, periosteal fibroblasts; OB, osteoblasts; OCY, osteocytes; L-NAME, N^G-Nitro-L-Arginine Methyl Ester. *Significant effect of PFF, $p < 0.05$.*

in osteocytes by more than 40% compared with static cultures (Figure 4.2 B), but it did not affect caspase-3 gene expression in osteoblasts or periosteal fibroblasts (Figure 4.2 B). PFF did not affect p53 gene expression in osteocytes, osteoblasts, or periosteal fibroblasts (Figure 4.2 C). PFF for 1 hr upregulated c-Jun gene expression in osteoblasts by 2.3-fold and in periosteal fibroblasts by 2.8-fold, but not in osteocytes (Figure 4.2 D).

To study a possible interrelationship between enhanced NO production resulting from PFF treatment and apoptosis-related gene expression in osteocytes, cells were subjected to 1 hr PFF in the presence of L-NAME. Addition of L-NAME to the culture medium during exposure to PFF blocked the upregulating effect of PFF on Bcl-2 gene expression in osteocytes, but did not affect Bcl-2 gene expression in osteoblasts or periosteal fibroblasts (Figure 4.3 A). L-NAME also blocked the downregulating effect of PFF on caspase-3 gene expression in osteocytes, but did not affect caspase-3 gene expression in osteoblasts or periosteal fibroblasts (Figure 4.3 B). Inhibition of NO production by L-NAME did not affect p53 gene expression in mechanically loaded osteocytes, osteoblasts, or periosteal fibroblasts (Figure 4.3 C). PFF stimulated c-Jun gene expression in the presence of L-NAME in osteocytes by 2.9-fold, in osteoblasts by 3.9-fold, and in periosteal fibroblasts by 5.1-fold (Figure 4.3 D).

DISCUSSION

We found that PFF stimulates Bcl-2 gene expression and inhibits caspase-3 gene expression, but did not alter p53 and c-Jun gene expression in osteocytes. Inhibition of NO synthesis by L-NAME prevented the PFF-mediated changes in Bcl-2 and caspase-3 gene expression in osteocytes. This suggests that NO is, at least in part, responsible for the loading-induced inhibition of osteocyte apoptosis via an effect on apoptosis-related gene expression.

The survival gene Bcl-2 is the first member of a gene family participating in the control of apoptosis[21]. In osteocytes, 1 hr PFF

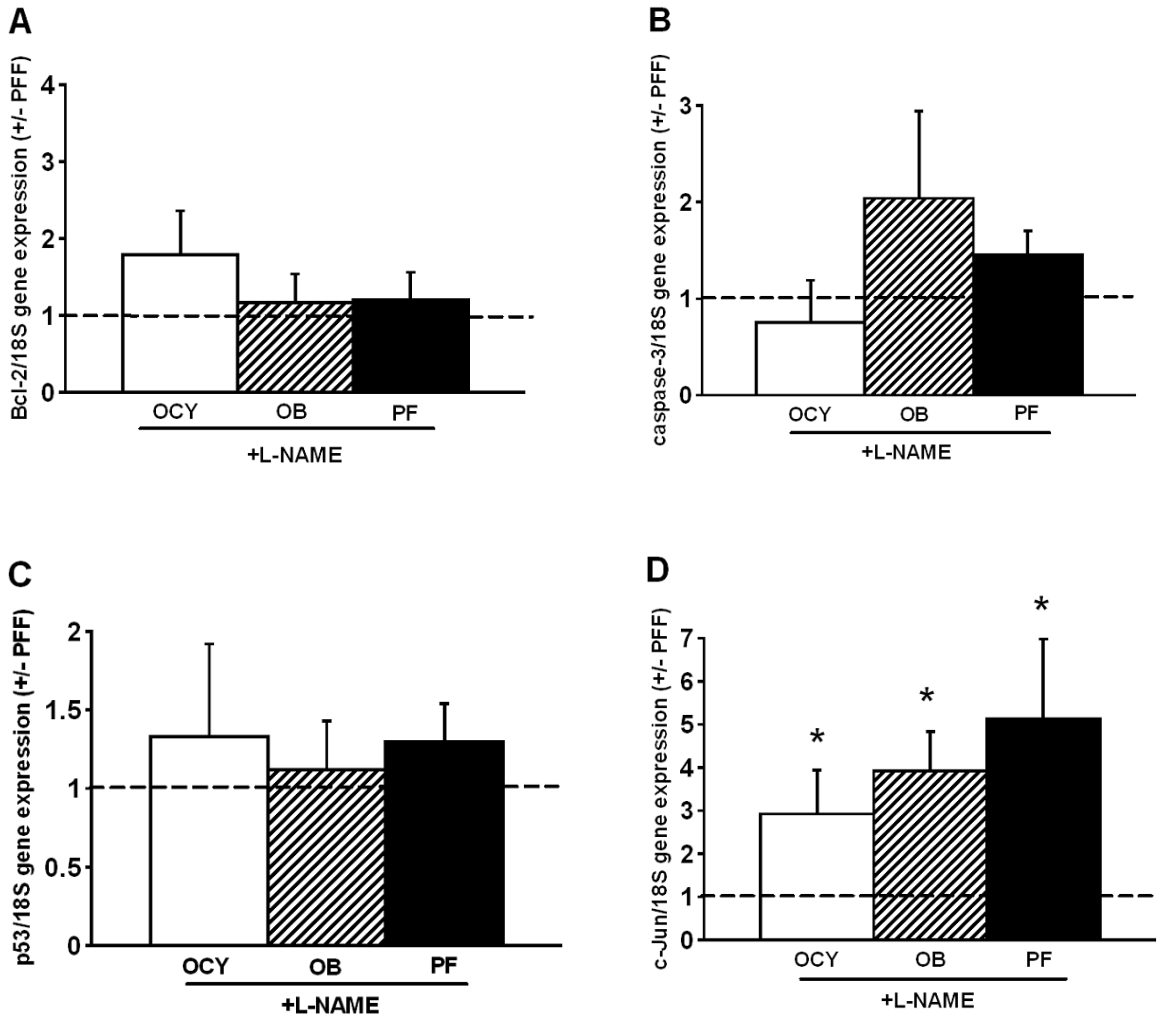

Figure 4.3 *Effect of pulsating fluid flow on Bcl-2, caspase-3, p53, and c-Jun gene expression in osteocytes, osteoblasts, and periosteal fibroblasts in the presence of L-NAME. (A) Bcl-2 gene expression in the presence of L-NAME. Addition of 1 mM L-NAME to the culture medium during exposure to PFF blocked the upregulating effect of PFF on Bcl-2 gene expression in osteocytes, but not in osteoblasts or periosteal fibroblasts. (B) Caspase-3 gene expression in the presence of L-NAME. L-NAME blocked the downregulating effect of PFF on caspase-3 gene expression in osteocytes, but not in osteoblasts and periosteal fibroblasts. (C) p53 gene expression in the presence of L-NAME. L-NAME did not affect p53 gene expression in osteocytes, osteoblasts, or periosteal fibroblasts. (D) c-Jun gene expression in the presence of L-NAME. L-NAME upregulated c-Jun gene expression in osteocytes (2.9-fold), osteoblasts (3.9-fold), and periosteal fibroblasts (5.1-fold). Values, obtained from 3-5 osteocyte, and 5-6 osteoblast and periosteal fibroblast experiments, are expressed as mean ± SEM of PFF-treated-over-control ratios (+/- PFF). Dashed line, +/- PFF = 1 (no effect). PFF, pulsating fluid flow; PF, periosteal fibroblasts; OB, osteoblasts; OCY, osteocytes; L-NAME, N^G-Nitro-L-Arginine Methyl Ester. *Significant effect of PFF, $p < 0.05$.*

upregulated Bcl-2 gene expression, which was NO-mediated. Enhanced Bcl-2 expression by NO has also been demonstrated in B-cells and endothelial cells[15,22], supporting our current findings. The PFF-mediated increase in Bcl-2 gene expression indicates that PFF inhibits osteocyte apoptosis via an increase in Bcl-2 protein expression. It should be noted that not Bcl-2 protein expression alone, but rather the ratio of Bcl-2 to Bax gene expression determines the susceptibility of cells to undergo apoptosis[23]. Unfortunately, we were unable to construct specific primers for Bax in chicken, and therefore we need to be cautious when interpreting our results.

Caspases can be thought of as the central executioners of the apoptotic pathway[24,25]. We observed that caspase-3 gene expression is downregulated by PFF in osteocytes, and that this effect is mediated by NO. This suggests that PFF-induced NO production inhibits osteocyte apoptosis in part by reducing caspase-3 expression. In a previous study, we showed that 1 hr PFF reduced caspase-3/7 activity in TNF-α-treated osteocytes[17]. Inhibition of NO synthesis by L-NAME prevented the PFF-mediated reduction in caspase-3/7 activity, suggesting that PFF-mediated NO release by osteocytes is, at least in part, responsible for the loading-induced inhibition of osteocyte apoptosis[17]. In line with these findings, it has been shown that NO inhibits apoptosis in endothelial cells via inhibition of caspase-3 activity[26].

No alterations in p53 gene expression were observed after PFF, and p53 gene expression remained unchanged when NO production was blocked. This suggests that the inhibitory effect of mechanical loading on osteocyte apoptosis is independent of p53 gene expression. c-Jun, a signal-transducing transcription factor of the AP-1 family normally implicated in cell cycle progression, differentiation, and cell transformation, is also linked to apoptosis[27,28]. Increased c-Jun activity is sufficient to trigger apoptotic cell death in NIH 3T3 fibroblasts[27]. Here, we show that PFF stimulates c-Jun gene expression in osteoblasts and periosteal fibroblasts, but not in osteocytes. Since no increase in NO production was observed in PFF-stimulated periosteal fibroblasts and osteoblasts, the PFF-induced increase in c-Jun gene expression in these cells is likely to be independent of NO production. Furthermore, we

previously found that PFF does not induce apoptosis in osteoblasts and periosteal fibroblasts. It is therefore unlikely that the increased c-Jun gene expression in these cells is linked to apoptosis.

Our results might offer, at least in part, an explanation for the complex process of orthodontic tooth movement. This process involves two major steps, i.e. resorption of existing bone by osteoclasts on the pressure side, and formation of new bone by osteoblasts on the tension side[3]. The question of why osteoclasts resorb bone matrix on the pressure side remains unexplained. On the pressure side, lowering of normal strain on the periodontal ligament occurs[29], which likely results in local stasis of extracellular fluid in the bone canalicular network, a lack of fluid shear stress on the osteocytes, and reduced NO production[13]. NO rapidly causes retraction of osteoclasts from their support[11]. Lack of loading-induced NO production on the pressure side might thus lead to a lack of inhibition of osteoclast activity. Based on our current findings, we propose that osteocytes enter apoptosis as a result of insufficient NO production due to insufficient fluid flow in their canaliculi. This hypothesis is supported by the observation that apoptotic osteocytes are present at the pressure side in rat alveolar bone[30]. Osteoclasts are attracted by apoptotic osteocytes[31], resulting in bone resorption. Evidence that osteoclastic attack is directed towards apoptotic osteocytes has been reported in the growing skeleton[31], as well as in relation to bone renewal[32] and under pathological conditions[33].

On the tension side, increased strain results in increased fluid flow in the canaliculi, which stimulates osteocytes to produce NO thereby maintaining osteocyte viability, and osteoblasts to produce new bone. The NO inhibitor L-NAME inhibits orthodontic tooth movement in rats[34], which might be explained by decreased bone formation on the tension side. These mechanisms, attraction of osteoclasts towards the pressure side and maintaining osteocyte viability at the tension side, can explain the mechanically meaningful behaviour of osteoclasts during orthodontic tooth movement. The increased NO production by osteocytes after mechanical stimulation by PFF modulates apoptosis-related gene expression suggesting that NO is a mediator of the

mechanical effects in bone, leading to bone remodeling such as occurs after orthodontic load.

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CHAPTER 5

OSTEOCYTES SUBJECTED TO FLUID FLOW INHIBIT OSTEOCLAST FORMATION AND BONE RESORPTION

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ABSTRACT

Bone has the capacity to alter its mass and structure to its mechanical environment. Osteocytes are the predominant bone cells and it is generally accepted that the osteocytes are the professional mechanosensors of bone. A strain-derived fluid flow through the lacuno-canalicular porosity seems to mechanically activate them, resulting in the production of signaling molecules such as nitric oxide (NO). We hypothesize that mechanically stimulated osteocytes modulate osteoclast formation and activity via soluble factors, thus affecting bone resorption.

Osteocytes, osteoblasts, and periosteal fibroblasts were isolated from fetal chicken calvariae via enzymatic digestion. The periosteal fibroblasts were obtained from the periostea. Osteocytes were separated from osteoblasts by immunomagnetic separation. Cells were mechanically stimulated for 1 hr with pulsating fluid flow (PFF, 0.70 ± 0.30 Pa) at 5 Hz, or kept under static conditions. Conditioned medium was collected after 60 min. The effect of conditioned medium on osteoclastogenesis was tested on mouse bone marrow cells in the presence of macrophage colony stimulating factor and receptor activator of NF-κB ligand. After 6 days of culture, osteoclast formation and bone resorption was determined.

Osteocytes subjected to 1 hr pulsating fluid flow produced conditioned medium that inhibited the formation of osteoclasts. For osteoblast PFF-conditioned medium, such effect was, to a lesser extent, also observed, but not for periosteal fibroblast PFF-conditioned medium. Furthermore, PFF-treated osteocytes, but not osteoblasts or periosteal fibroblasts, produced conditioned medium that resulted in a decreased bone resorption. The NO synthase inhibitor N^G-Nitro-L-Arginine Methyl Ester (L-NAME) attenuated the inhibitory effects of osteocyte PFF-conditioned medium on osteoclast formation and resorption.

We conclude that osteocytes subjected to PFF inhibit osteoclast formation and resorption via soluble factors, and the release of these factors was at least partially dependent on activation of an NO pathway in osteocytes in response to PFF. Thus, the osteocyte appears to be

more responsive to PFF than the osteoblast or periosteal fibroblast regarding to the production of soluble factors affecting osteoclast formation and bone resorption.

Key words: osteocyte, osteoblast, osteoclast, osteoclast formation, osteoclast activity, bone resorption, mechanical loading, fluid flow, *in vitro*

INTRODUCTION

Osteocytes are the predominant bone cells and are considered to be the orchestrators of bone adaptation to loading. Osteocytes are surrounded by bone, and are in contact with neighbouring osteocytes via long slender cell processes, located in canaliculi, which are filled with pericellular interstitial fluid[1]. These cell processes contact not only neighboring osteocytes but also cells lining the bone surface such as osteoblasts, bone lining cells, and osteoclasts. This three-dimensional network of interconnected cells is present throughout bone, and it has been suggested that this osteocyte network with its accompanying lacuno-canalicular porosity is the site of mechanosensing in bone[2-5]. Mechanotransduction then includes the translation, by osteocytes, of canalicular flow into cell signals that can recruit osteoclasts and osteoblasts[3].

When bone is loaded, pericellular fluid is squeezed through the three-dimensional network, resulting in a fluid flow. This flow results in a strain-driven movement of interstitial fluid through the canaliculi and along the osteocyte processes. Pericellular fluid may be critical in transmitting soluble mediators from osteocytes to other bone cells to produce a desired response to mechanical stimulation. Osteocytes subjected to pulsating fluid flow regulate osteoblast proliferation and differentiation[6], and proliferation of bone marrow mesenchymal stem cells[7]. In-depth examination of the effects of conditioned medium from osteocytes on osteoclast formation and activity can help to expand our understanding of the soluble factors released into the pericellular fluid in response to osteocyte loading.

Previous studies have demonstrated that shear stress produced by the flow of pericellular fluid is essential to the transduction of mechanical stimuli to the mechanosensitive osteocyte[5,8]. We and others have shown that in cell culture experiments, osteocytes produce high levels of nitric oxide (NO) in response to fluid shear stress[9,10]. This messenger molecule is involved in the regulation of many physiological processes, such as vascular relaxation[11], neurotransmission, platelet aggregation, and immune regulation[12]. In bone, it has been shown that

NO mediates adaptive bone formation *in vivo*[13] and protects osteocytes against apoptosis[14]. Furthermore, endogenously produced NO is essential for osteoclast activity[15]. High levels of NO have been shown to rapidly reduce the osteoclast spread area followed by retraction of the cells from the tissue culture support[16]. Inhibition of NO production however increases osteoclastogenesis and osteoclast activity[17]. Thus, we suggest that NO may modulate osteoclast formation and activity.

In the clinic, remodeling of bone is crucial during orthodontic tooth movement. This process involves two major steps: resorption of existing bone by osteoclasts on the pressure side and formation of new bone by osteoblasts on the tension side[18]. The question of how resorbing osteoclasts find their way through the pre-existing bone matrix remains unexplained. On the pressure side during orthodontic tooth movement, lowering of normal strain from the functioning periodontal ligament occurs[19], which might result in local stasis of extracellular fluid in the canalicular network of bone[20,21], a lack of fluid shear stress on the osteocytes, and reduced NO production. Furthermore, the strain-derived flow of interstitial fluid through the periodontal ligament is decreased, and the release of NO is inhibited[22]. Fluid stasis in combination with reduced NO production then might induce osteoclast formation and bone resorption.

We hypothesized that fluid flow has an effect on osteoclast formation and function[23], likely by the osteocytes and either or not through NO, but until now, no studies have shed light on this issue. The aim of the present study was to clarify the extent to which soluble factors released by osteocytes, osteoblasts, or periosteal fibroblasts in response to mechanical simulation affect osteoclast formation and bone resorption. To validate that NO may modulate osteoclast formation and bone resorption, the release of NO was inhibited by N^G-Nitro-L-Arginine Methyl Ester (L-NAME).

MATERIALS AND METHODS

Isolation and culture of osteocytes, osteoblasts, and periosteal fibroblasts

Fetal chicken calvarial cells were isolated as described earlier[1]. Briefly, calvariae were aseptically dissected from 18-day-old chicken fetuses. Periostea were removed and digested with 1 mg collagenase type I/ml (Sigma, St. Louis, MO, USA) phosphate-buffered saline (PBS), to isolate periosteal fibroblasts. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in alpha minimum essential medium (α-MEM; Gibco, Paisley, Scotland) supplemented with 2% chicken serum (Gibco), 200 µg/ml glutamine (Sigma), 50 µg/ml gentamicin sulfate (Sigma), 50 µg/ml L-ascorbic acid (Merck, Darmstadt, Germany), and 1 mg/ml D-glucose (Merck). The calvariae without periosteum were sequentially digested with 1 mg/ml collagenase, and 4 mM EDTA in PBS, to obtain a mixed population of osteoblasts and osteocytes, which was cultured as described above for the periosteal fibroblasts.

The next day, osteocytes were separated from osteoblasts by immunomagnetic separation, using the osteocyte-specific monoclonal antibody mAb OB 7.3[24]. To remove the magnetic beads from the osteocytes, 200 U/100 µl DNAse (Invitrogen, Breda, The Netherlands) was added for 15 min. Osteocytes did not proliferate. Osteocytes, osteoblasts, and periosteal fibroblasts were then seeded at the same density on polylysine-coated (50 mg/ml; poly-L-lysine hydrobromide, mol wt 15-30 x 10^4; Sigma) glass slides, in culture medium, and left to attach overnight.

Pulsating fluid flow

Osteocytes, osteoblasts, and periosteal fibroblasts were either or not subjected to 1 hr of pulsating fluid flow as described previously[2]. Briefly, PFF was generated by pumping 13 ml of culture medium through a parallel-plate flow chamber containing the bone cells. The cells were subjected to a 5 Hz pulse with a mean shear stress of 0.7 Pa, a pulse amplitude of 0.3 Pa and a peak shear stress rate of 8.4 Pa/sec. Stationary control cultures were kept in a petridish under similar conditions as the experimental cultures, i.e. at 37°C in a humidified

atmosphere of 5% CO_2 in air. Conditioned medium was collected after 1 hr of PFF or static culture.

Inhibition of nitric oxide production

NO release was inhibited by adding 1 mM N^G-Nitro-L-Arginine Methyl Ester (L-NAME; Sigma). The NO inhibitor was added to the culture medium of osteocytes, osteoblasts, and periosteal fibroblasts during exposure to PFF. L-NAME did not directly affect osteoclast formation and bone resorption (data not shown).

Administration of nitric oxide

NO was administered by adding 5 µM *S*-nitroso-*N*-acetylpenicillamine (SNAP; Sigma). The NO donor was added to the culture medium of osteocytes under static conditions.

Osteoclast formation

Osteoclast formation was assessed as described earlier[25]. Briefly, five weeks old male mice were killed with a peritoneal injection of a lethal dose of Euthesate (8 mg sodium pentobarbital per mouse; Sanofi Santé Animale Benelux B.V., Maassluis, The Netherlands). Tibiae were removed and cleaned of soft tissue and ground in a mortar with α-MEM (Gibco) supplemented with 5% fetal calf serum (HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B (antibiotic antimyotic solution; Sigma), and heparin (170 IE/ml; Leo Pharmaceutical Products B.V., Weesp, The Netherlands). The cell suspension was aspirated through a 21-gauge needle and filtered over a 100 µm pore size Cell Strainer filter (Falcon/Becton Dickinson, Franklin Lakes, NJ, USA). Cells were washed twice in culture medium, centrifuged (5 min, 200*xg*), and seeded in 96-well flat-bottom tissue culture-treated plates (Costar, Cambridge, MA, USA) or seeded on 650-µm-thick bovine cortical bone slices at a density of 1 x 10^5 cells.

Cells were cultured in 150 µl culture medium containing 30 ng/ml recombinant murine M-CSF (R&D systems, Minneapolis, MI, USA), 20 ng/ml recombinant murine RANKL (RANKL-TEC, R&D systems), and 50%

CM. Filter-sterilized CM from osteocytes, osteoblasts, and periosteal fibroblasts treated with or without pulsating fluid flow was obtained as described above, and was added (1:1, volume/volume) to the fresh culture medium. Culture media and conditioned media were replaced after 3 days. After 6 days of culture, cells were fixed in 4% formaldehyde in PBS for 10 min. Fixed cells were washed with PBS, and stained for tartrate-resistant acid phosphatase (TRACP) according to manufacturer's instruction (Sigma). The number of TRACP-positive multinucleated cells (3 or more nuclei per cell) was counted using a Leica DM IL microscope (Leica, Wetzlar, Germany) equipped with a 20x objective.

Bone resorption

After 6 days of culture, cells present on the bovine cortical bone slices were removed using 0.25 M NH_4OH. The slices were washed in water, incubated in a saturated alum ($KAl(SO_4)_2 12H_2O$) solution, washed in water, and stained with Coomassie brilliant blue[26]. The area of individual resorption pits were measured using a computerized XY-tablet (Qwin, Leica). Three fields per bone slice were examined, and percentage of total bone resorption per bone slice was calculated.

Statistics

Statistical analysis was performed by student's *t*-test. Differences were considered significant when $p < 0.05$ (two-tailed).

RESULTS

Osteocyte PFF-conditioned medium inhibits osteoclast formation

Mouse bone marrow cells cultured for 6 days in the presence of RANKL and M-CSF resulted in the formation of TRACP-positive multinucleated cells (Figure 5.1 A). Osteocytes subjected to 1 hr PFF produced conditioned medium (CM) that significantly inhibited the formation of osteoclasts, as assessed by the number of TRACP-positive multinucleated cells after 5 days of culture in a mouse bone marrow

osteoclastogenesis assay (Figure 5.1 B, C). Osteocyte PFF CM reduced the formation of osteoclasts by 75% compared to osteocyte static CM. For osteoblast PFF CM, a similar inhibition was observed, but to a lesser extent (Figure 5.1 B, C). Osteoblast PFF CM reduced the formation of osteoclasts by 55% compared to osteoblast static CM. For periosteal fibroblast PFF CM an inhibition of osteoclast formation was also observed (49%), but this inhibition was not statistically significant (Figure 5.1 B, C).

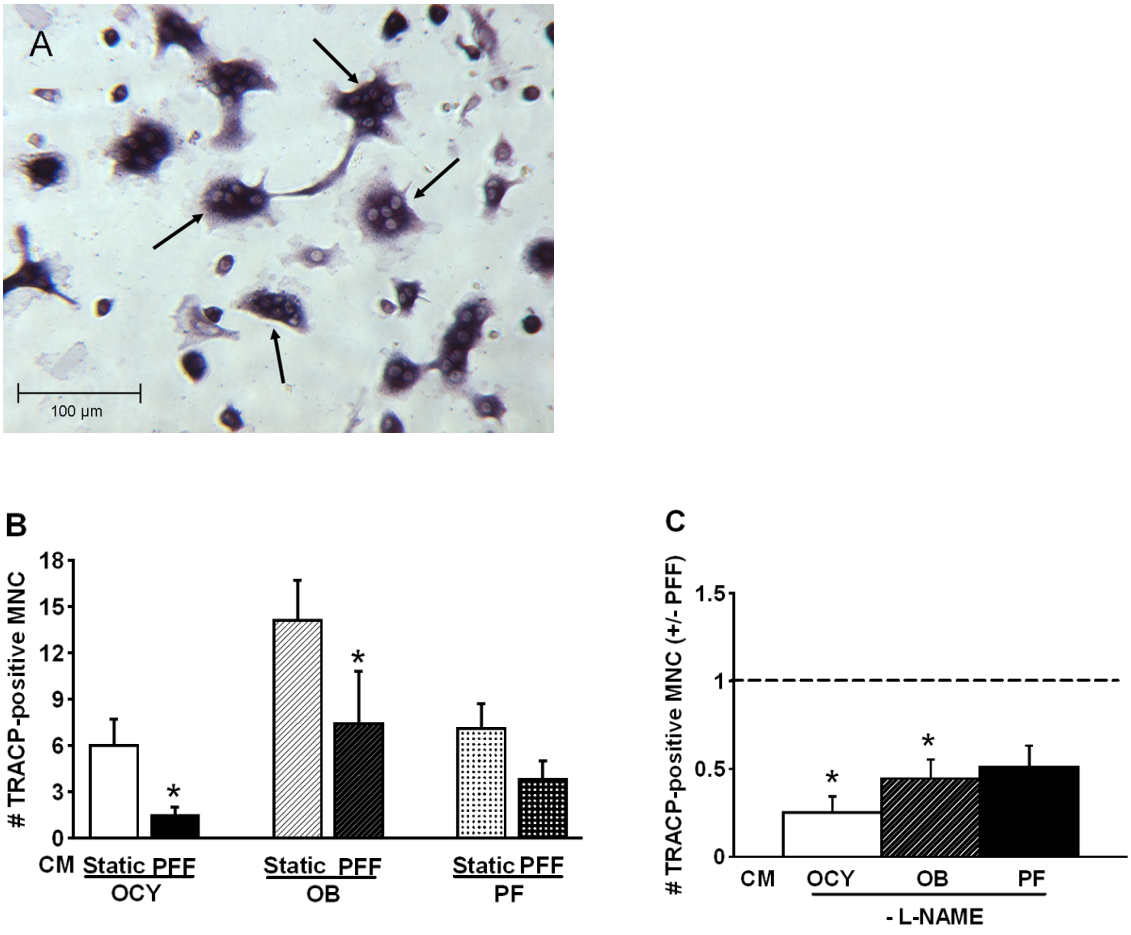

Figure 5.1 *Effect of conditioned medium obtained from osteocytes, osteoblasts, and periosteal fibroblasts treated with or without pulsating fluid flow on osteoclast formation. (A) TRACP-positive multinucleated cells (arrows) after 6 days of culture in the presence of osteocyte static CM. Magnification: 20x. (B) Osteoclast formation expressed as absolute values in the absence of L-NAME, as assessed as the number of TRACP-positive multinucleated cells (TRACP-positive MNC). (C) Osteoclast formation expressed as PFF-treated-over-control ratios (+/- PFF) in the absence of L-NAME. Osteocyte PFF CM reduced the formation of osteoclasts compared to osteocyte static CM (-75%). For osteoblast PFF CM a similar inhibition was observed, but to a lesser extent (-55%). For periosteal*

*fibroblast PFF CM an inhibition of osteoclast formation was also observed, but this inhibition (-49%) was not statistically significant. Data, obtained from 4 independent experiments with n = 2 for each experiment, are expressed as mean ± SEM (B), or are expressed as mean ± SEM of PFF-treated-over-control ratios (+/- PFF) (C). Dashed line, +/- PFF = 1 (no effect). OCY, osteocytes; OB, osteoblasts; PF, periosteal fibroblasts; PFF, pulsating fluid flow; CM, conditioned medium; L-NAME, N^G-Nitro-L-Arginine Methyl Ester. * Significant effect of PFF, p < 0.05.*

L-NAME attenuates the inhibitory effects of osteocyte PFF CM on osteoclast formation

Addition of 1 mM L-NAME, an inhibitor of nitric oxide synthase, to the culture medium during exposure to PFF blocked the inhibitory effect of osteocyte PFF CM on the formation of osteoclasts (Figure 5.2). The number of osteoclasts formed in the presence of osteocyte PFF CM was similar to that formed by osteocyte static CM (Figure 5.2). Addition of L-NAME during PFF exposure furthermore attenuates the inhibitory effects of osteoblast PFF CM and periosteal fibroblast PFF CM on osteoclast formation (Figure 5.2).

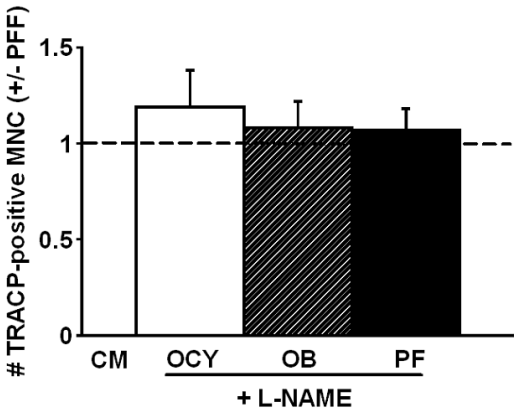

Figure 5.2 *Effect of conditioned medium obtained from osteocytes, osteoblasts, and periosteal fibroblasts treated with or without pulsating fluid flow on osteoclast formation in the presence of L-NAME. Addition of 1 mM L-NAME to the culture medium during exposure to PFF blocked the inhibitory effect of osteocyte PFF CM on the formation of osteoclasts. The number of osteoclasts generated in the presence of osteocyte PFF CM was similar to that generated by osteocyte static CM. Addition of L-NAME during PFF exposure attenuated the inhibitory effects of osteoblast PFF CM and periosteal fibroblast PFF CM on osteoclast formation. Values, obtained from 4 independent experiments with n = 4 for each experiment, are expressed as mean ± SEM of PFF-treated-over-control ratios (+/- PFF). Dashed line, +/- PFF = 1 (no effect). OCY, osteocytes; OB, osteoblasts; PF, periosteal fibroblasts; PFF, pulsating fluid flow; CM, conditioned medium; L-NAME, N^G-Nitro-L-Arginine Methyl Ester. * Significant effect of PFF, p < 0.05.*

SNAP inhibits osteoclast formation

The conditioned medium obtained from osteocytes under static conditions in the presence of 5 µM SNAP, a nitric oxide donor, completely blocked the formation of TRACP-positive multinucleated cells (Figure 5.3). SNAP also directly affected osteoclast formation and resorption to a comparable extent (data not shown).

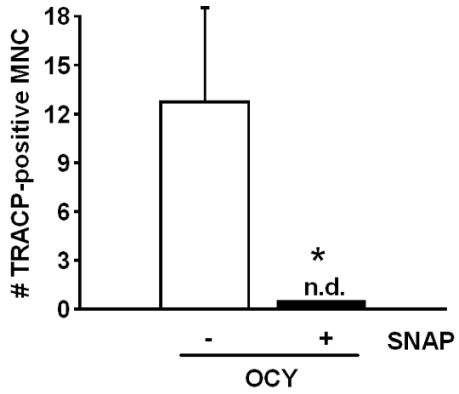

Figure 5.3 *Effect of conditioned medium obtained from osteocytes under static conditions in the presence of SNAP. Addition of 5 µM SNAP to the culture medium during static conditions dramatically attenuated the formation of TRACP-positive multinucleated cells (TRACP-positive MNC). Values, obtained from 1 experiment with n = 4, are expressed as mean ± SEM. OCY, osteocytes; SNAP: S-nitroso-N-acetylpenicillamine; n.d.: not detectable. * Significant effect of SNAP, p < 0.05.*

Osteocyte PFF CM inhibits osteoclastic bone resorption

Mouse bone marrow cells cultured for 6 days in the presence of RANKL and M-CSF on bovine cortical bone slices resulted in the formation of resorption pits (Figure 5.4 A) that were clearly visible after removal of the cells from the bone surface. Osteocytes subjected to 1 hr of PFF produced CM that significantly inhibited osteoclastic bone resorption (Figure 5.4 B). Analysis of bone resorption revealed that the average formed resorption area by osteoclasts cultured with osteocyte static CM was 4.5 ± 1.7% (mean ± SEM, 4 independent experiments with n = 8), and the average resorption area under the influence of osteocyte PFF CM was 1.9 ± 0.5% (mean ± SEM, 4 independent experiments with n = 8) (Figure 5.4 B). For osteoblast PFF CM and periosteal fibroblast PFF CM no inhibition of osteoclastic bone resorption was observed (Figure 5.4 B).

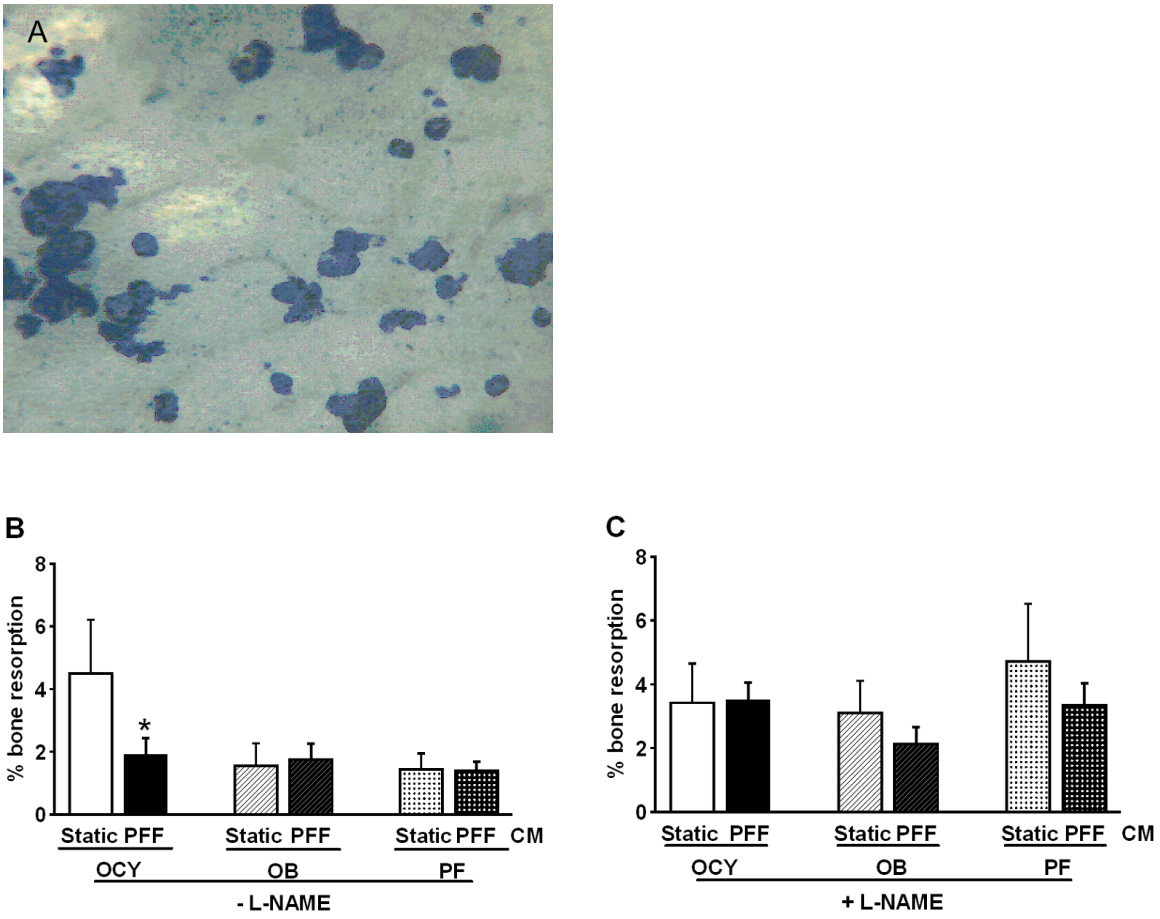

Figure 5.4 *Effect of conditioned medium obtained from osteocytes, osteoblasts, and periosteal fibroblasts with or without pulsating fluid flow on osteoclastic bone resorption. (A) Resorption pit (dark blue area) in a bone slice on which mouse bone marrow cells were cultured in the presence of osteocyte static CM. (B) Osteoclastic bone resorption in the absence of L-NAME. Osteocyte PFF CM reduced bone resorption compared to osteocyte static CM. For osteoblast PFF CM and periosteal fibroblast PFF CM, no inhibition of osteoclastic bone resorption was observed. (C) Osteoclastic bone resorption in the presence of L-NAME. Addition of 1 mM L-NAME to the culture medium during exposure to PFF blocked the inhibitory effect of osteocyte PFF CM on bone resorption by osteoclasts. The percentage of bone resorption by osteocyte PFF CM was similar to that formed by osteocyte static CM. Addition of L-NAME during PFF exposure had no effect on the CM obtained from osteoblasts and periosteal fibroblasts on osteoclastic bone resorption. Values, obtained from 4 independent experiments with n = 8 (B, C) for each experiment, are expressed as mean ± SEM. OCY, osteocytes; OB, osteoblasts; PF, periosteal fibroblasts; PFF, pulsating fluid flow; CM, conditioned medium; L-NAME, N^G-Nitro-L-Arginine Methyl Ester. * Significant effect of PFF, $p < 0.05$.*

L-NAME attenuates the inhibitory effects of osteocyte PFF CM on osteoclastic bone resorption

Addition of 1 mM L-NAME to the culture medium during exposure to PFF blocked the inhibitory effects of osteocyte PFF CM on osteoclastic bone resorption (Figure 5.4 C). The percentage of bone resorption by osteoclasts in the presence of osteocyte PFF CM was similar to that caused by osteoclasts cultured with osteocyte static CM (Figure 5.4 C). Addition of L-NAME during PFF exposure had no effect on the CM obtained from osteoblasts and periosteal fibroblasts on osteoclastic bone resorption (Figure 5.4 C).

DISCUSSION

In response to mechanical loading, osteocytes communicate with each other and other types of cells including osteoblasts, bone lining cells, and osteoclasts in bone through the canalicular network in which nutrients and other soluble factors are transported by fluid flow[27]. Considering the close physical proximity of osteocytes to osteoblasts, bone lining cells, and osteoclasts, it is highly plausible that soluble factors produced by osteocytes act in a paracrine manner to affect these cells. We have shown earlier that conditioned medium from osteocytes subjected to pulsating fluid flow inhibits proliferation but stimulates differentiation of osteoblasts *in vitro*[6], and that conditioned medium from osteocytes stimulates the proliferation of bone marrow mesenchymal stem cells and their differentiation into osteoblasts[7]. The present study was conducted to elucidate the effects of soluble mediators released by PFF-stimulated osteocytes on the formation and activity of osteoclasts. Pulsating fluid flow was examined in this work since this type of mechanical stimulus is similar to the *in vivo* forces experienced by osteocytes in the lacuno-canalicular system of bone[4].

We found that the treatment of osteocytes with PFF produced conditioned medium that significantly inhibited the formation of osteoclasts *in vitro*. This suggests that a soluble mediator released by osteocytes, and to lesser extent also by osteoblasts, in response to PFF

is responsible for inhibiting osteoclast formation. Furthermore, osteocyte PFF CM inhibited osteoclastic bone resorption. This suggests that in response to mechanical loading soluble factors produced by osteocytes signal osteoclasts to decrease bone resorption.

Osteocytes kept under static conditions represent a situation of no mechanical stress and no fluid shear stress along the cell membrane. Osteocyte static CM increased osteoclastic bone resorption. This suggests that in a disuse situation[28], bone resorption increases.

To our knowledge, this work examines for the first time the effects of conditioned medium from primary osteocytes on osteoclast formation and bone resorption. A previous study has shown that mechanical stimulation of fetal mouse bone *in vitro* inhibits osteoclastic bone resorption[29]. This study used an intermittent compressive force, while the present study used a pulsating fluid shear stress regime. Conditioned medium from primary osteocytes has not been studied before because primary osteocytes are notoriously difficult to isolate in large numbers. The effects of conditioned medium from fetal mouse cartilaginous long bones and the MLO-Y4 osteocyte-like cell line in the presence or absence of mechanostimulation on osteoclast formation and activity have been investigated[29-32]. The conditioned medium from MLO-Y4 cells inhibited osteoclastic bone resorption[30] without affecting the number of mature osteoclasts[30,31]. This information has made significant contribution to our knowledge of the effects of conditioned medium of these cells, but it is limited by the fact that an osteocyte-like cell line was used instead of primary osteocytes. Primary osteocytes *in vivo*, in contrast to cell lines, do not proliferate. In addition, mechanical stimulation of osteocytes does produce conditioned medium whose effects are significantly different from those in the absence of mechanical loading, as we have shown in this study.

Furthermore, we investigated if NO can modulate osteoclast formation and bone resorption. NO is a particularly interesting soluble mediator released by osteocytes in that it has been implicated in the catabolic response of bone to mechanical loading[23,33]. NO also prevents osteocyte apoptosis[14], and since osteoclastic attack is directed towards apoptotic osteocytes[34], NO can prevent bone resorption. Previous

studies in our laboratory have demonstrated that mechanical stimulation by PFF produces a vigorous response by osteocytes as evidenced by an increase in NO release[9,10], and addition of 1 mM L-NAME during PFF inhibits the PFF-mediated NO synthesis[10]. Here we show that the inhibition of NO production by L-NAME prevented the inhibiting effect of conditioned medium from osteocytes subjected to PFF on osteoclast formation and bone resorption. Furthermore, our findings demonstrate that conditioned medium obtained from SNAP-treated osteocytes under static conditions elicited an inhibitory effect on osteoclast formation, possibly by inducing apoptosis of bone marrow cells, as also was reported elsewhere[35]. These results suggest that NO is a mediator of mechanical effects in bone, leading to inhibition of osteoclast formation and bone resorption.

In a previous study, we found that fluid flow rapidly enhanced the release of NO in primary human bone cell cultures, and that eNOS is the likely isoenzyme involved in this response[36]. The increased eNOS mRNA expression after PFF treatment is likely related to the shear stress responsive element in the promoter of eNOS[36]. It is likely that eNOS is also responsible for the PFF-induced NO production in chicken osteocytes, but this still needs to be confirmed in another study.

Fluid flow-induced shear stress as well as NO donors were shown to decrease osteoclastogenesis through RANKL and OPG signaling[37,38]. Cytokine-induced NO also inhibited bone resorption by inducing apoptosis of osteoclast progenitors and suppressing osteoclast activity, and it was suggested that immature cells in the bone marrow compartment are most sensitive to NO-induced apoptosis[35]. These studies corroborate our findings that NO is a potent inhibitor of bone resorption with effects on both osteoclast formation and activity. Further experiments focusing on identifying the specific soluble mediators in addition to NO that are involved in bone remodeling as a result of mechanical loading will add to our understanding of the molecular mechanisms involved in bone mechanotransduction.

Our results might offer, at least in part, an explanation for the complex process of orthodontic tooth movement, in which many cells and molecules are involved. During orthodontic tooth movement,

periodontal ligament cells are stretched or compressed[39], and molecules such as NO are found in the gingival tissue[40]. We suggest that on the pressure side local stress shielding due to decreased functioning of the periodontal ligament occurs[19], which causes fluid stasis in the canaliculi of the osteocyte[21], a lack of fluid shear stress on the osteocytes, and reduced NO production. Furthermore, the strain-derived flow of interstitial fluid through the periodontal ligament is decreased on the pressure side, and the release of NO by periodontal ligament fibroblasts is reduced[22]. Fluid stasis in combination with reduced NO production then induces osteoclast formation and activity, resulting in bone resorption. On the tension side, increased strain likely results in increased fluid flow, which stimulates osteocytes and periodontal ligament fibroblasts to produce NO, thereby inhibiting osteoclast formation and activity.

In summary, osteocytes subjected to one-hour PFF produced CM that significantly inhibited the formation of osteoclasts. For osteoblast PFF CM such effect was, to a lesser extent, also observed, and for periosteal fibroblast PFF CM the effect was not significant. Furthermore, treatment of osteocytes with PFF produced CM that inhibited osteoclast activity. For osteoblast and periosteal fibroblast PFF CM, no such effect was observed. These inhibitory effects were, at least in part, mediated by NO. Thus, the osteocyte appears to be more responsive to PFF than the osteoblast or periosteal fibroblast with respect to the production of soluble factors affecting osteoclast formation and bone resorption. This suggests a regulatory role for osteocytes in osteoclast formation and bone resorption during bone remodeling such as occurs after application of an orthodontic load.

ACKNOWLEDGEMENTS

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CHAPTER 6

ORTHODONTIC FORCE STIMULATES eNOS AND iNOS IN RAT OSTEOCYTES

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ABSTRACT

Mechanosensitive osteocytes play a key role in bone remodeling. Nitric oxide, an important regulator of bone remodeling, is produced by osteocytes through the activity of constitutive endothelial nitric oxide synthase (eNOS) or inducible nitric oxide synthase (iNOS). We hypothesize that these enzymes regulate the tissue response to orthodontic force, and therefore we investigated eNOS and iNOS expression in osteocytes during orthodontic force application.

The upper rat molars were moved mesially by NiTi-coil springs (10 cN, 120 hrs), using a split-mouth design. Immunohistochemical staining revealed that in the tension area, eNOS-positive osteocytes increased from 24 hrs on, while iNOS-positive osteocytes remained largely constant. In the compression area, iNOS-positive osteocytes increased already after 6 hrs, while eNOS-positive osteocytes increased after 24 hrs. This suggests that eNOS mediates bone formation in the tension area, while iNOS mediates inflammation-induced bone resorption in the compression area. Both eNOS and iNOS seem to regulate bone remodeling during orthodontic force application.

Key words: osteocyte, eNOS, iNOS, nitric oxide, orthodontic force

INTRODUCTION

Orthodontic tooth movement is characterized by sequential reactions of the periodontal tissues to biomechanical forces[1]. Orthodontic research mostly focuses on osteoblasts, osteoclasts, and fibroblasts in the periodontal ligament (PDL)[1-4]. However, little is known about the role of osteocytes in alveolar bone during orthodontic tooth movement. It is assumed that physiological changes in periodontal tissues during tooth movement influence activity, metabolism, and communication of alveolar bone osteocytes[5].

Osteocytes are the predominant bone cells and are well-equipped to orchestrate bone adaptation to loading[6]. Osteocytes are in contact with neighboring osteocytes, osteoblasts, and bone lining cells via a canalicular network that is filled with pericellular fluid[7]. It has been suggested that this three-dimensional network is the site of mechanosensing in bone[8-11]. Mechanotransduction then includes the translation, by osteocytes, of canalicular flow into cellular signals that can recruit osteoclasts and osteoblasts[9].

Nitric oxide (NO) is an important regulator of the response of bone to mechanical stress, and is produced through the activity of constitutive endothelial nitric oxide synthase (eNOS) or inducible nitric oxide synthase (iNOS)[12]. Several *in vitro* studies have shown that NO is rapidly increased in response to mechanical stress in bone cells[13,14]. It mediates adaptive bone formation[15], and protects osteocytes against apoptosis[16,17]. Furthermore, endogenously produced NO mediates osteoclast activity[18]. High levels of NO reduce osteoclast activity[19], while inhibition of NO production increases osteoclastogenesis and osteoclast activity[20,21].

eNOS is the predominant nitric oxide synthase (NOS) isoform in bone and produces small quantities of NO. It is expressed on a constitutive basis in osteocytes and chondrocytes[22,23]. eNOS knockout mice display severe skeletal abnormalities[24]. eNOS-derived NO from osteocytes likely plays a regulatory role in the adaptive response to mechanical loading[23,25].

iNOS has been detected in osteocytes, osteoclasts, and

chondrocytes, and is expressed in response to inflammatory cytokines[12]. Once induced, iNOS produces large amounts of NO. Inflammatory conditions are associated with bone resorption and activation of iNOS in bone cells[26], which suggests that iNOS-mediated NO production stimulates inflammation-induced bone resorption[18].

We hypothesize that eNOS and iNOS expression in osteocytes changes locally in alveolar bone in response to orthodontic force. Therefore, in the present study we investigated eNOS and iNOS expression in osteocytes during orthodontic force application in a rat model.

MATERIALS AND METHODS

Experimental design

Forty six-week-old male Wistar rats were acclimatized for at least one week before the start of the experiment, and were housed under normal laboratory conditions with powdered laboratory chow (Sniff, Soest, The Netherlands) and water *ad libitum*. The rats were divided into eight groups, with five animals per group. A split-mouth design was used, in which at random the left or right side of the maxilla was chosen as experimental side, and the controlateral as control side. Ethical permission was obtained from the Radboud University Nijmegen Medical Centre.

The orthodontic appliance has been described earlier[27]. Briefly, a preformed stainless steel ligature wire enclosing all three maxillary molars was bonded (Clearfil SE Bond, Kuraray Europe GmbH, Düsseldorf, Germany) on the experimental side. This allowed the movement of the three molars as one unit. For anchorage, a transverse hole was drilled through the alveolar bone and both maxillary incisors at the mid-root level. A stainless steel ligature wire was put through the hole, and a custom-made activated 10 cN Sentalloy® close coil spring (GAC, New York, USA) was attached to it and to the molar block. By this design, tipping movement was almost completely prevented, and the three molars together moved bodily mesially. All treatments were performed

under general anaesthesia (FFM-mix, i.p. 2.8 ml/kg, which contained 6.8 mg/kg Fluanisone, 0.1 mg/kg Fentanyl, and 3.4 mg/kg Midazolam).

After 6, 12, 24, 36, 48, 72, 96, and 120 hrs of force application, one group of five rats was killed by inhalation anaesthesia (5% Isoflurane for induction and 2-3% for maintenance), and perfused through the left heart ventricle with 4% freshly prepared paraformaldehyde in phosphate buffered saline (PBS). The maxillae were dissected and fixed in 4% paraformaldehyde for 24 hrs, decalcified with 10% EDTA, and embedded in paraffin. The period of decalcification was variable (2-4 weeks), and was checked radiographically.

Selection of sections

Serial para-sagittal sections of 5 µm were cut from paraffin-embedded tissue blocks, mounted on Superfrost Plus slides (Menzel-Gläser; Braunschweig, Germany), and stained with haematoxylin-eosin for general tissue survey. For immunohistochemical staining, sections that contained the radicular pulp of 3-6 roots of the maxillary molars were selected. For determination of eNOS and iNOS-positive osteocytes, two sections that were at least 25 sections apart were stained. Six-to-twelve roots per rat were evaluated.

Immunohistochemistry

Sections were deparaffinated and rehydrated. The sections were pre-incubated with 0.1% trypsin in Tris/HCl buffer, treated with 3% H_2O_2 in PBS, and heated to 70°C in 10 mM citrate buffer in a microwave oven for 10 min. Non-specific binding of the secondary antibody was blocked by pre-incubation with 10% normal donkey serum (Biomeda, Foster City, USA) in PBS. Thereafter, sections were incubated at 4°C overnight with rabbit polyclonal antibodies to eNOS (used at dilution of 1:100) (Abcam, Cambridge, MA, USA) or iNOS (used at dilution of 1:400) (Abcam). Both antibodies do not cross-react with other NOS isoforms. After washing, sections were incubated with a biotin-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, Westgrove, PA, USA) for 45 minutes. The sections were then incubated with ABC peroxidase (Vector Laboratories, Burlingham, CA, USA), which was visualized by a

diaminobenzidine/ nickel solution (Sigma, St. Louis, USA). The staining was enhanced with 0.5% $CuSO_4$ in 0.9% NaCl. As negative control, sections were incubated with control rabbit IgG replacing the primary antibody.

Quantification of eNOS and iNOS-positive osteocytes

The number of eNOS and iNOS-positive osteocytes was quantified in the mesial and distal bone area along the selected roots at the experimental and control side. Since the three maxillary molars were moved mesially as one unit, the mesial PDL along the selected roots at the experimental side were considered as compression area, and the distal PDL as tension area. The mean number of eNOS or iNOS-positive osteocytes within the bone along these areas (mesial or distal) for each experimental and control side was calculated. Each quantification was blindly performed by the same investigator (SDT). To standardize the data, the number of positive osteocytes located less than 60 µm from the bone surface were quantified along the entire root length. To enable corrections for differences in osteocyte density, their numbers were determined in 12 randomly chosen sections. The mean total number of osteocytes per mm^2 was 1049 ± 209 (mean ± SD), and no differences were found in the mesial and distal bone area at the experimental and control side. Therefore, it was decided to express the data as number of positive osteocytes/mm^2 of bone area.

Statistical analysis

To determine the random intra-individual error for cell counting, 8 randomly chosen sections were recounted and Dahlberg's equation was calculated. The random intra-individual error was 5.25 positive osteocytes/mm^2, which is acceptable. Differences in the number of eNOS and iNOS-positive osteocytes in the mesial and distal bone area along the selected roots at experimental and control side over time were assessed by one-way ANOVA. In case of significance, Tukey's multiple comparison test was used. Differences were considered significant when $p < 0.05$.

RESULTS

General tissue survey

All rats were healthy throughout the experiment. The direction of the orthodontic force by the activation spring remained parallel to the maxilla. After 6 hrs of force application, the three molars had bodily moved to the mesial within their sockets, hence the mesial PDL was compressed and the distal PDL widened (Figure 6.1 A). Onset of hyalinization of the compressed PDL was found after 24 hrs of orthodontic force application.

Immunohistochemical staining

Staining demonstrated eNOS and iNOS-positive osteocytes in the entire mesial and distal bone area along the selected roots at the experimental and control side. Positive osteocytes were evenly distributed along the entire root length close to the bone surface (Figure 6.1 A, B, C).

Control side

Equal numbers of eNOS as well as iNOS-positive osteocytes were present in both the mesial and distal bone area at the control side. No significant changes were found in the mesial or the distal bone area along the selected roots at the control side over time. Therefore, the mean number of eNOS and iNOS-positive osteocytes in the mesial and distal bone area at the control side were taken as baseline value for the experimental side.

Experimental side

Compression area, eNOS

In the compression area, the baseline for the number of eNOS-positive osteocytes was 69.8 ± 5.8 (mean ± SEM; Figure 6.2 A). A significant effect on the number of eNOS-positive osteocytes was found over time ($p < 0.01$). At 24 and 48 hrs, the number of eNOS-positive osteocytes was significantly higher than baseline (Figure. 6.2 A, B). After 48 hrs however, the number decreased.

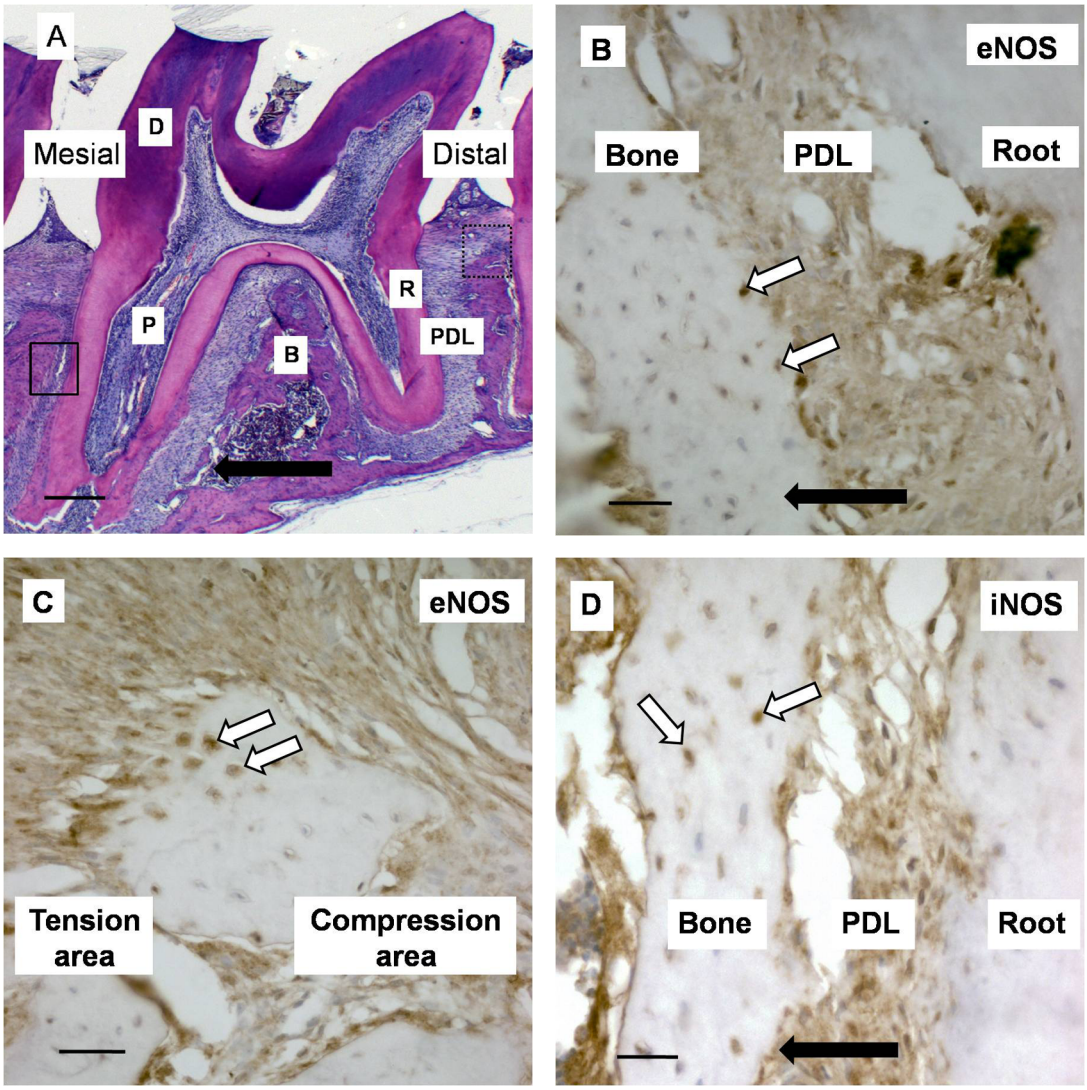

Figure 6.1 *Immunohistochemical staining for endothelial nitric oxide synthase and inducible nitric oxide synthase during orthodontic force application in a rat model. (A) Overview of an orthodontically moved second rat molar after 6 hrs of force application. Alveolar bone (B), dentin (D), periodontal ligament (PDL), pulp (P), and root (R) are indicated. The solid frame indicates a field of interest, viewed in more detail in figure 6.1 B and 6.1 D. The dotted frame indicates a field of interest, viewed in more detail in figure 6.1 C. Bar = 400 µm. (B) Immunohistochemical staining for endothelial nitric oxide synthase (eNOS) after 36 hrs of force application in the compression area. Bar = 40 µm. (C) Immunohistochemical staining for eNOS after 72 hrs of force application in the tension and compression area. Positive stained cells are present in the tension area, but not in the compression area. Bar = 40 µm. (D) Immunohistochemical staining for inducible nitric oxide synthase (iNOS) after 6 hrs of force application in the compression area. Bar = 40 µm. Black arrow: direction of force application. White arrow: positive osteocytes.*

Tension area, eNOS

In the tension area, the baseline for the number of eNOS-positive osteocytes was 46.6 ± 3.5 (mean ± SEM, Figure 6.2 C). A significant effect on the number of eNOS-positive osteocytes was found over time ($p < 0.01$). From 24 hrs on, the number of eNOS-positive osteocytes was significantly higher than baseline, and increased by 2.3 to 2.9-fold (Figure 6.2 C, D).

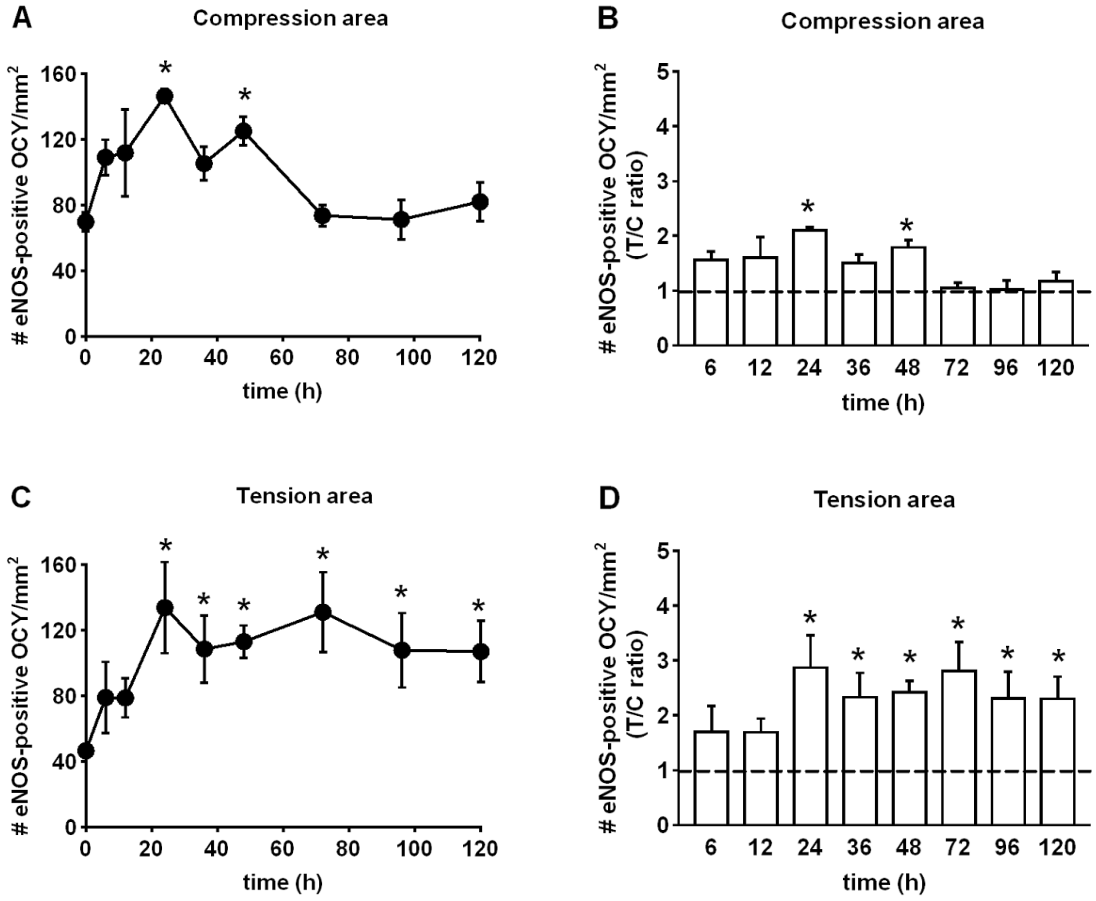

Figure 6.2 *Number of eNOS-positive osteocytes in the compression area (A, B) and tension area (C, D) during orthodontic force application in a rat model. Values (mean ± SEM) are expressed as the number of eNOS-positive osteocytes (OCY) per mm^2 bone area (A, C), or as orthodontic force-treated-over-control (T/C) ratios (B, D). Dashed line, +/- T/C ratio = 1 (no effect). Values are based on five rats per time point, whereby osteocyte numbers from 6-12 roots were averaged to obtain the mean number of eNOS-positive osteocytes per area. * Significantly different from baseline (A, C) or significant effect of orthodontic force (B, D), p < 0.05.*

Compression area, iNOS

In the compression area, the baseline for the number of iNOS-positive osteocytes was 20.5 ± 2.3 (mean ± SEM, Figure 6.3 A). A significant effect on the number of iNOS-positive osteocytes was found over time ($p < 0.01$). The number of iNOS-positive osteocytes was significantly higher than baseline from 6 to 48 hrs, and increased by 3.0 to 4.1-fold (Figure 6.3 A, B). Thereafter, the number decreased to baseline (Figure 6.3 A, B).

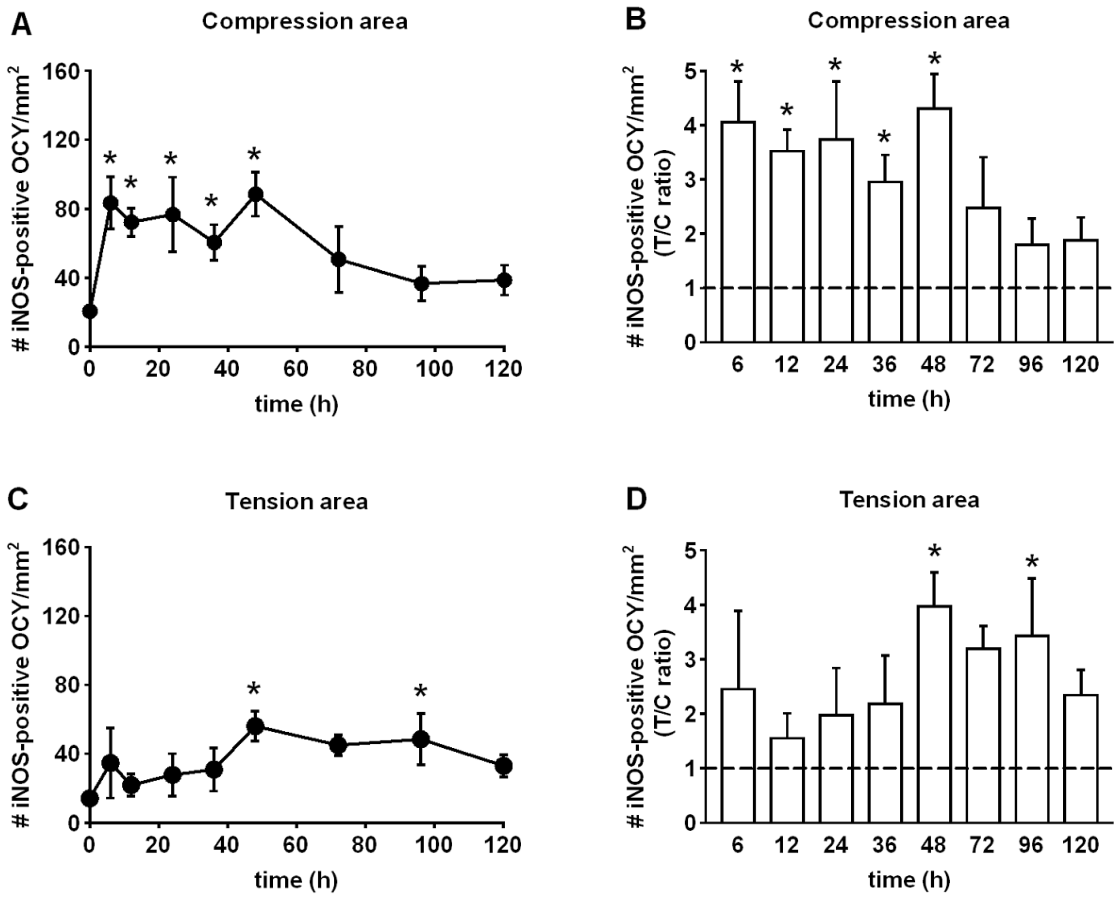

Figure 6.3 *Number of iNOS-positive osteocytes in the compression area (A, B) and tension area (C, D) during orthodontic force application in a rat model. Values (mean ± SEM) are expressed as the number of iNOS-positive osteocytes (OCY) per mm² bone area (A, C), or as orthodontic force-treated-over-control (T/C) ratios (B, D). Dashed line, T/C ratio = 1 (no effect). Values are based on five rats per time point, whereby the osteocyte number from 6-12 roots were averaged to obtain the mean number of iNOS-positive osteocytes per area. * Significantly different from baseline (A, C) or significant effect of orthodontic force (B, D), p < 0.05.*

Tension area, iNOS

In the tension area, the baseline for the number of iNOS-positive osteocytes was 14.1 ± 2.1 (mean ± SEM; Figure. 6.3 C). A significant effect on the number of iNOS-positive osteocytes was found over time ($p < 0.01$). Post-hoc analysis however, only showed significant differences with baseline at 48 and 96 hrs (Figure 6.3 C, D).

DISCUSSION

In the present study, a standardized force of 10 cN was used to move three molars as one block in an *in vivo* rat model for orthodontic tooth movement. Since rat molars are approximately 50 times smaller than human premolars, this force is comparable with a force of 150-175 cN applied to one human molar. We found that iNOS-positive osteocytes appeared mainly in the compression area, while in the tension area eNOS-positive osteocytes were present.

Increased levels of gingival eNOS and iNOS have been reported during orthodontic treatment in humans, indicating a role for eNOS and iNOS in the early phase of orthodontic tooth movement[28]. *In vivo* studies suggest that NO is an important mediator in the response of periodontal tissue to orthodontic force, and an increase in NO production leads to increased tooth movement[29-31]. Other *in vitro* investigations have reported changes in NO levels in PDL fibroblasts in response to loading, suggesting that NO is a mediator of mechanical stress[32]. However, little is known on the expression of eNOS and iNOS in osteocytes during orthodontic force application. Therefore, the aim of the present study was to investigate eNOS and iNOS expression in osteocytes during orthodontic force application in a rat model. As NO production by NOS is a rather quick process after mechanical stimulation, we limited the experimental period to 120 hrs.

We found that positive osteocytes were located close to the bone surface. Using finite element analysis, it has been described that strain is induced within the bony matrix upon physiological loading, and this strain results in a flow of fluid up to a depth of 100 µm, with a maximum

flow at a depth of 30 μm[33]. In cell culture experiments, osteocytes produce high levels of NO in response to fluid shear stress in vitro[13,23]. We suggest that orthodontic force results in a strain within the bone, which give rise to a flow of fluid resulting in the production of NO by osteocytes. This explains that positively stained osteocytes were found close to the bone surface.

The orthodontic force reversed the physiological distal drift of the molars[34] to a mesial migration. At the control side, the number of eNOS and iNOS-positive osteocytes in the mesial and distal bone area did not change during the experimental period. Therefore, the mean number of eNOS and iNOS-positive osteocytes in the mesial and distal bone area at the control side were taken as baseline for the experimental side. The baseline for the number of eNOS-positive osteocytes was higher than that for iNOS-positive osteocytes during normal physiological drift, both in the mesial and distal bone area. This confirms results from other studies, showing that although both eNOS and iNOS are expressed in bone, eNOS is the prevailing isoform[22,23,26].

In the compression area the number of iNOS-positive osteocytes increased already after 6 hrs. This might be related to the rapid onset of hyalinization of the PDL and subsequent production of inflammatory cytokines[1,35] , resulting in inflammation induced bone resorption in the compression area[36]. Interestingly, mediators such as interleukin-1β and prostaglandin E_2 show a peak in crevicular fluid at 24 hrs after orthodontic force application[37]. We found that after 48 hrs, the number of iNOS-positive osteocytes returned to baseline. This suggests that iNOS-mediated NO production by osteocytes triggers inflammation-induced bone resorption in the compression area. The number of eNOS-positive osteocytes in the compression area showed a comparable time course, although the increase started only after 24 hrs.

In the tension area, the number of eNOS-positive osteocytes increased after 24 hrs, and remained high during the entire experimental period. This may be related to the stretching of the PDL fibers at the tension side, resulting in increased bone strain[38]. The number of iNOS-positive osteocytes however was not influenced at the tension side. iNOS is expressed in response to inflammatory cytokines

and it has been described that tensile strains of low magnitude inhibits induction of pro-inflammatory cytokines in human periodontal ligament cells[39]. This suggests that bone formation in the tension area seems to be stimulated by eNOS rather than by iNOS.

In summary, iNOS-positive osteocytes appeared mainly in the compression area, while in the tension area eNOS-positive osteocytes were present. This suggests that eNOS mediates bone formation in the tension area, while iNOS mediates inflammation-induced bone resorption in the compression area. Both eNOS and iNOS seem to be important regulators of bone remodeling during orthodontic force application.

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CHAPTER 7

GENERAL DISCUSSION

GENERAL DISCUSSION

Throughout life bones adapt their mass, shape, and trabecular architecture according to the mechanical environment in order to achieve an optimal resistance to mechanical failure with a minimum use of material. It has been well-demonstrated that increased mechanical loading stimulates, and unloading decreases bone mass, mineral content, and bone matrix protein production[1-5]. This process is known as functional adaptation of bone, and is obtained during the complicated process of bone remodeling[2,6]. The bone remodeling process is executed by bone resorbing osteoclasts and bone depositing osteoblasts, which are orchestrated by mechanosensing osteocytes[7-9].

This thesis examined the role of mechanical loading in various aspects of bone adaptation such as occurs during orthodontic tooth movement, at the cellular level. Osteocyte apoptosis likely regulates bone remodeling by attracting osteoclasts. Nitric oxide (NO) is an important signaling molecule in response to mechanical loading, and is produced by osteocytes through the activity of constitutive endothelial nitric oxide synthase (eNOS) and/or inducible nitric oxide synthase (iNOS). We hypothesized that the osteocytes play a key role in the adaptation of bone to mechanical loading, and that this bone adaptation process is at least partially regulated by NO. We demonstrated that the number of osteocytes embedded in the bone matrix depends on gender, and differs between healthy and osteoporotic subjects (Chapter 2). Mechanical stimulation by fluid shear stress inhibited osteocyte apoptosis (Chapter 3 and 4), and the inhibition of osteocyte apoptosis is mediated by NO (Chapter 3 and 4). Furthermore, we demonstrated that osteocytes subjected to pulsating fluid flow (PFF) inhibit osteoclast formation and bone resorption (Chapter 5). Finally, we found that during orthodontic force application, iNOS-positive osteocytes appeared mainly in the compression area, while in the tension area eNOS-positive osteocytes appeared (Chapter 6).

The purpose of this general discussion is to relate the experimental findings of the earlier chapters to bone adaptation such as occurs during orthodontic tooth movement.

Osteocytes as orchestrators of bone adaptation

Osteocytes constitute over 90-95% of the bone cell population[10]. Stellate-shaped osteocytes reside in the lacuno-canalicular network within the bone matrix, with the cell bodies occupying the lacunae and the cell processes radiating into the canaliculi. Nearly 50-60 cell processes, which are generally less than 0.5 µm in diameter, radiate from individual cell bodies in different directions[11]. Via their cell processes osteocytes form a syncytium with the surrounding osteocytes and the cells lining the bone surface[12]. Anatomically, osteocytes are ideally placed in the bone matrix to perform mechanosensation[13-15]. The manner whereby osteocytes sense the strains of minimized matrix has been considered in the light of very small strains in the bone during daily loading, as compared to muscle tissue[16,17]. This has led to the concept of strain-derived canalicular fluid flow, or its derivative, fluid drag force, as the physical mediator of mechanosensing by osteocytes in bone tissue[16,18,19]. Several studies based on animal models as well as cell cultures support this concept[2,9,20-25]. Together, they suggest that the osteocyte network with its accompanying lacuno-canalicular porosity is the site of mechanosensing in bone tissue. Mechanotransduction then includes the translation, by osteocytes, of canalicular flow into cell signals that can recruit osteoclasts and osteoblasts. Osteocyte-ablated mice are resistant to unloading-induced bone loss, providing evidence for the role of osteocytes in mechanotransduction[26].

Osteocytes act in close cooperation with osteoblasts during their incorporation in the bone matrix[27], and osteocyte density is suggested to reflect the result of the bone remodeling process[28]. Hence, increased knowledge of osteocyte density will likely contribute to a better understanding of bone biology, and we investigated the relationship between osteocyte density, bone remodeling parameters, gender, and osteoporosis (Chapter 2). Our results demonstrated that the number of osteocytes embedded in the bone matrix depends on gender, and differs between healthy and osteoporotic subjects. Osteoporotic patients showed reduced bone turnover and changed bone architecture, which is characteristic for osteoporosis and the results are consistent with impaired osteoblast function in osteoporotic patients (Chapter 2).

Osteocytes are notoriously difficult to isolate in large numbers due to their deep seated anatomical location in the bony matrix, and therefore few studies have investigated the effect of mechanical loading on primary osteocytes, but used the MLO-Y4 osteocyte-like cell line instead[29-31]. We have studied the effect of mechanical loading on primary osteocytes[32-34] (Chapter 3, 4, and 5) and we have subjected the osteocytes to a pulsating fluid shear stress regime (Chapter 3, 4, and 5), while other studies have proposed different kinds of mechanical loading regimes such as stretching[29] and steady fluid shear stress[30]. However, a pulsating fluid shear stress (with a 5 Hz pulse with a mean shear stress of 0.7 Pa, a pulse amplitude of 0.3 Pa, and a peak shear stress rate of 8.4 Pa/sec) mimics the manner by which loading of whole bones is thought to be conveyed to the osteocytes *in vivo*[8,16,35].

Osteocytes communicate with each other and other types of cells including osteoblasts, bone lining cells, and osteoclasts in bone through the canalicular network in which nutrients and other soluble factors are transported by fluid flow[36,37]. Considering the close physical proximity of osteocytes to osteoblasts, bone lining cells, and osteoclasts, it is highly plausible that soluble factors produced by osteocytes act in a paracrine manner to affect these cells. Unloaded osteocytes produce factors such as sclerostin that directly promote osteoclastic bone resorption[38]. It has been shown earlier that conditioned medium from osteocytes subjected to PFF inhibits proliferation but stimulates differentiation of osteoblasts *in vitro*[39], and that conditioned medium from osteocytes stimulates the proliferation of bone marrow mesenchymal stem cells and their differentiation into osteoblasts[40]. Chapter 5 was conducted to elucidate the effects of soluble mediators released by PFF-stimulated osteocytes on the formation and activity of osteoclasts. We found that osteocytes subjected to PFF inhibit osteoclast formation and resorption via soluble factors, and the release of these factors was at least partially dependent on activation of an NO pathway in osteocytes in response to PFF. This suggests a regulatory role for osteocytes in osteoclast formation and bone resorption during bone remodeling.

Osteocyte apoptosis

The importance of apoptosis, or programmed cell death, for normal tissue development and turnover in biology is increasingly recognized. Apoptosis is conserved in animal species and represents critical steps of the regulated development of multicellular organisms. For example, in the embryonic development of the limb, programmed cell death plays a role in sculpturing the shape of the digits[41]. Apoptosis also plays a role in the growth plate of long bones, where programmed cell death occurs in hyperthrophic chondrocytes, which are removed by osteoclasts as part of the replacement of cartilage by bone[42]. Besides its' role in development, apoptosis operates in adult organisms to maintain cell and tissue homeostasis. Evidence indicates that insufficient apoptosis can manifest as cancer[43], while accelerated cell death occurs in acute and chronic degenerative diseases such as atherosclerosis[44].

During bone adaptation, local disuse of bone leads to a local lack of canalicular fluid flow and therefore to a lack of fluid shear stress. This lack of fluid shear stress induces osteocyte apoptosis[45]. In endothelial cells, shear stress by blood flow inhibits cell apoptosis[46]. The inhibition of cell apoptosis might importantly contribute to the functional integrity of the endothelial cell layer and thereby inhibit damage to the arterial wall, which is a key event for initiating atherosclerotic lesion development[47]. Furthermore, apoptosis of endothelial cells attracts macrophages that phagocytose the dying cells[48].

We propose that such a mechanism also operates in bone. We hypothesized that osteocytes are protected against apoptosis by fluid shear stress (Chapter 3 and 4), and that apoptotic osteocytes could be the cellular signal for osteoclast recruitment resulting in bone resorption[7]. Evidence that osteoclastic attack is directed towards apoptotic osteocytes has been reported[49-52], suggesting a key regulatory role for osteocyte apoptosis in bone remodeling. We investigated if mechanical loading by PFF affected apoptosis in chicken osteocytes, osteoblasts, and periosteal fibroblasts (Chapter 3 and 4), and which apoptosis-related genes were altered in their expression (Chapter 4). We found that fluid shear stress inhibits apoptosis specifically in tumor necrosis factor-α-treated osteocytes (Chapter 3), and that PFF stimulates

Bcl-2 gene expression and inhibits caspase-3 gene expression, but did not alter p53 and c-Jun gene expression in osteocytes (Chapter 4). These results suggest a regulatory role for osteocyte apoptosis in osteoclastic bone resorption during bone remodeling.

Apoptosis can be induced by a variety of stimuli including the activation of death receptors. Tumor necrosis factor-α (TNF-α) activates caspases, which are the main executioners of apoptosis[53,54]. Depending on the cell type, signaling pathways downstream of the death receptors can be modulated by different proteins such as Bcl-2, which has been shown to have an important regulatory function in osteocyte apoptosis. The mechanisms by which apoptotic cells might regulate osteoclastic activity are unknown, but several intriguing possibilities exist. Similar to macrophages, osteoclasts are attracted to apoptotic cells that expose phosphatidylserine on their outer cell surface[42,55,56]. Under static control conditions, osteocytes exposed phosphatidylserine on their cell membrane 9x more often than after treatment with a PFF[45]. Exposure of phosphatidylserine on osteocytic cell "fingers" might therefore be the signal that urges the osteoclasts to resorb bone. Furthermore, it has been suggested that osteocyte apoptosis could provide a targeting signal for resorption via DNA fragmentation products[49,51].

Nitric oxide

Mechanical loading of osteocytes initiates the production of signaling molecules like prostaglandins and NO. NO is produced when L-arginine is converted to L-citruline in the presence of constitutive eNOS and/or iNOS, molecular oxygen, NADPH, and other cofactors[57,58]. eNOS is widely expressed in bone on a constitutive basis, whereas iNOS is expressed in response to inflammatory cytokines[58]. In bone, NO has been shown to modulate the activity of bone forming osteoblasts and bone resorbing osteoclasts[59-62]. We and others have shown that in cell culture experiments, osteocytes produce high levels of NO in response to loading in the form of a physiologic fluid shear stress[23-25,57,61,63-65] and to a localized mechanical loading on the single cell level[36,66]. Loading-induced NO production in bone cells results from the activity of constitutive eNOS, a plasma membrane bound enzyme originally

believed to be specific for endothelium but later also found in osteocytes and osteoblasts[57,67,68]. Interestingly, eNOS is the NO producing enzyme isoform specifically involved in the cellular response to fluid shear stress, as it occurs in the endothelium of blood vessels in response to blood flow[69]. The finding that osteocytes also express eNOS, and that the bone cell enzyme is also activated by fluid shear stress, suggests that there may be common functions for eNOS in endothelium and osteocytes. In endothelial cells, NO production by eNOS in response to fluid flow plays a major role in preventing apoptosis[48,70]. In areas of reduced blood flow, as at the periphery of blood vessel bifurcations where atherosclerotic plaque accumulates, endothelial cell death occurs and it is believed to be caused by insufficient NO production as a result of insufficient fluid shear stress over the cells in that area of the vessel wall[46]. NO production in response to adequate shear stress protects the endothelial cells against apoptosis[70].

We proposed that such a mechanism also operates in bone, and that osteocytes are also protected against apoptosis by a basal amount of NO production under normal canalicular shear stress (Chapter 3 and 4). Conversely, during unloading osteocytes might enter apoptosis as a result of insufficient NO production due to insufficient fluid flow (Chapter 3 and 4). We found that inhibition of NO synthesis by N^G-Nitro-L-Arginine Methyl Esther (L-NAME) prevented the PFF-mediated downregulation of apoptosis in osteocytes (Chapter 3 and 4). Thus, the inhibition of osteocyte apoptosis by fluid flow is mediated by NO.

Since osteoclastic attack is directed towards apoptotic osteocytes[49], we suggested that NO can prevent bone resorption. We found that the inhibition of NO production by L-NAME prevented the inhibiting effect of conditioned medium from osteocytes subjected to PFF on osteoclast formation and bone resorption (Chapter 5). These results suggest that NO is a mediator of mechanical effects in bone, leading to inhibition of osteocyte apoptosis (Chapter 3 and 4), and of osteoclast formation and bone resorption (Chapter 5).

The precise mechanisms responsible for inhibition of apoptosis are not clear. It has been demonstrated that NO inhibits TNF-α-induced apoptosis via the inhibition of caspase-3 activation in human endothelial

cells[71], and that NO regulates the gene expression of caspase[72]. In B-cells, enhanced Bcl-2 expression by NO has been demonstrated[73] and the TNF-α-induced proteolytical cleavage of Bcl-2 in human endothelial cells is inhibited by NO[48]. A possible mechanism for the inhibition of osteoclast activity by NO is the modification of cathepsin K. Cathepsin K is highly expressed in osteoclasts and plays a key role in the bone resorption mechanism, since it degrades bone collagen. NO and several NO donors have been shown to inhibit the activity of cathepsin K[74].

Orthodontic tooth movement is characterized by sequential reactions of the periodontal tissues to biomechanical forces. We hypothesized that eNOS and/or iNOS regulate the tissue response to orthodontic force, and therefore we investigated eNOS and iNOS expression in osteocytes during orthodontic force application in a rat model (Chapter 6). We found that iNOS-positive osteocytes appeared mainly in the compression area, while in the tension area eNOS-positive osteocytes were present (Chapter 6). This suggests that eNOS mediates bone formation in the tension area, while iNOS mediates inflammation-induced bone resorption in the compression area. Both eNOS and iNOS seem to be important regulators of bone remodeling during orthodontic force application.

Implications for orthodontic tooth movement (Figure 7.1)

Our results might offer, at least in part, an explanation for the complex process of orthodontic tooth movement, in which many cells and cytokines are involved. This process involves two major steps, i.e. resorption of existing bone by osteoclasts at the site clinically referred to as “pressure side”, and formation of new bone by osteoblasts at the site clinically referred to as “tension side”[75-77]. Periodontal ligament cells are stretched or compressed[76], and cytokines such as TNF-α and interleukin-1 are produced[78,79]. The question of why osteoclasts resorb bone matrix at the “pressure side” remains unexplained. It is generally assumed that the coordinated cooperation of the osteoclasts and osteoblasts during bone adaptation is orchestrated by the osteocytes, which are able to respond to mechanical signals[8,13,80].

We suggest that together with TNF-α in the gingival sulcus,

osteocyte apoptosis is caused at the “pressure side” by local stress shielding due to decreased functioning of the periodontal ligament[78,81,82], which causes almost complete fluid stasis in the canaliculi of the osteocytes[83], a lack of fluid shear stress on the osteocytes, and reduced NO production[63]. As a result of insufficient NO production, osteocytes enter apoptosis via an increased caspase-3 gene expression and activity, and a decreased Bcl-2 gene expression. Osteoclasts are then attracted by apoptotic osteocytes[49], resulting in bone resorption and remodeling. Furthermore, lack of loading-induced NO production at the “pressure side” leads to a lack of inhibition of osteoclast activity, resulting in bone resorption. Finally, pro-inflammatory cytokines activation of the iNOS pathway in osteocytes, and NO derived from this pathway potentiates inflammation-induced bone resorption at the “pressure side”[84].

At the “tension side”, increased strain results in increased fluid flow in the canaliculi. Osteocytes receive enhanced fluid shear stress, and NO production will therefore be higher than normal. The high NO level will prevent osteocyte apoptosis and will maintain osteocyte viability via a decreased caspase-3 gene expression and activity, and an increased Bcl-2 gene expression. Furthermore, high NO levels will inhibit osteoclast formation and activity, and will promote the retraction and detachment of osteoclasts from the bone surface[62]. Finally, eNOS-derived NO from osteocytes likely regulates osteoblast activity and bone formation in response to mechanical loading at the “tension side”[58].

These mechanisms, attraction of osteoclasts towards the “pressure side” and maintaining osteocyte viability at the ”tension side”, can explain the mechanically meaningful behaviour of osteoclasts during orthodontic tooth movement, leading to bone remodeling.

Taken together, we suggest that the osteocytes respond to the opposite flow patterns, i.e. by facilitating osteoclastic activity at the “pressure side” where flow is low, and by inhibiting osteoclastic bone resorption at the “tension side” where flow is high. Osteoclast activity could thus be related to opposite flow patterns, while the mechanosensitive osteocytes are of crucial importance for orchestrating the remodeling process such as occurs after orthodontic load[6].

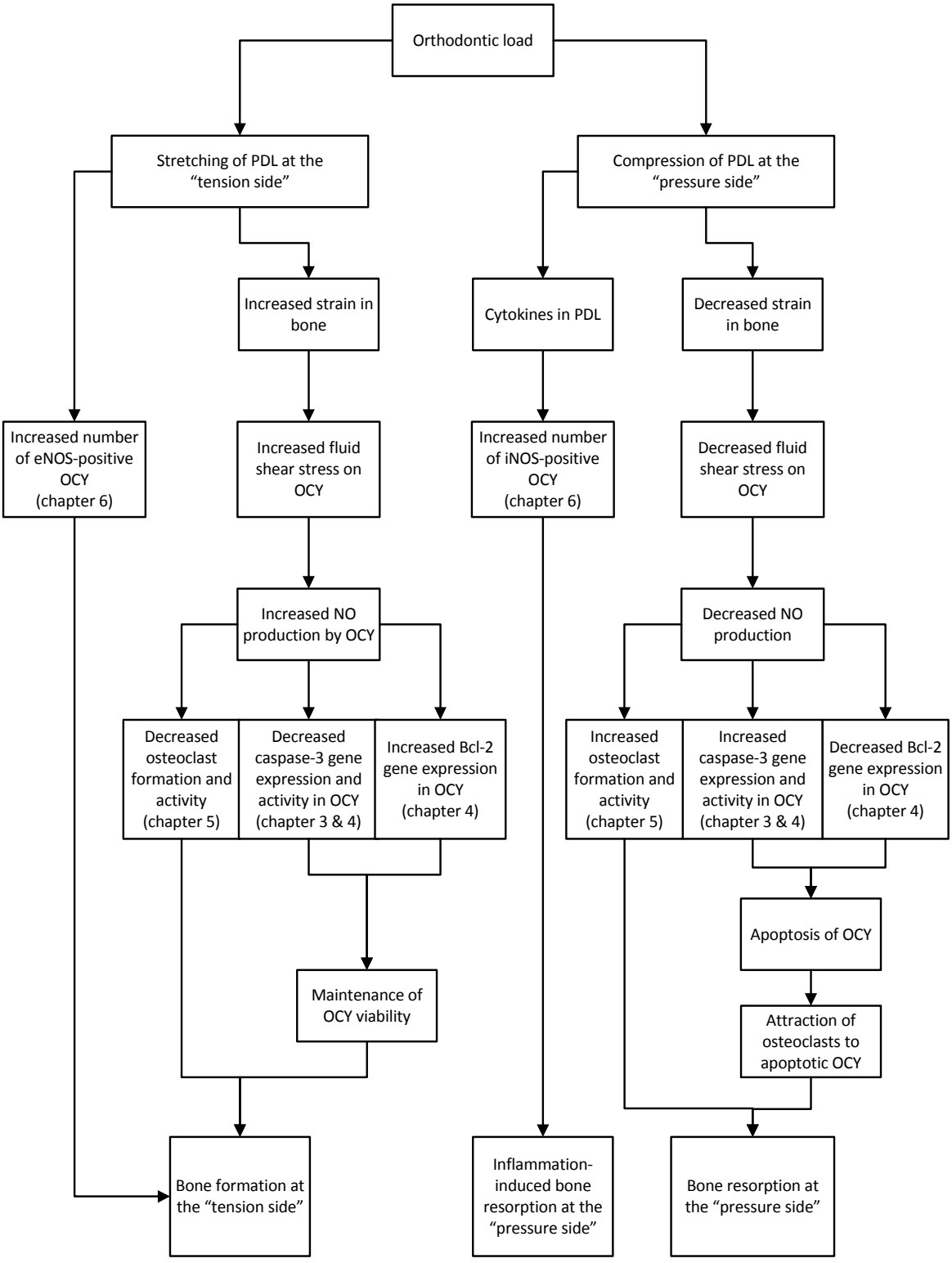

Figure 7.1 *Flowchart of the findings of this thesis, which might offer an explanation for the complex process of an orthodontic tooth movement, whereby an orthodontic load leads to bone formation at the "tension side" and bone resorption at the "pressure side". PDL, periodontal ligament; OCY, osteocytes; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NO, nitric oxide*

Conclusion

The number of osteocytes embedded in the bone matrix depends on gender, and differs between healthy and osteoporotic subjects. Osteoporotic patients showed reduced bone turnover and changed bone architecture, which is characteristic for osteoporosis, and the results are consistent with impaired osteoblast function in osteoporotic patients (Chapter 2). Mechanical loading by PFF of physiological magnitude reduced TNF-α-induced apoptosis in osteocytes (Chapter 3), and the inhibition of osteocyte apoptosis by PFF was regulated via alterations in Bcl-2 and caspase-3 gene expression (Chapter 4). This suggests a regulatory role for osteocyte apoptosis during bone remodeling. NO is, at least in part, responsible for the loading-induced inhibition of osteocyte apoptosis (Chapter 3 and 4). Treatment of osteocytes with PFF produced conditioned medium that significantly inhibited the formation of osteoclasts and osteoclastic bone resorption *in vitro* (Chapter 5). This suggests that a soluble mediator released by osteocytes in response to PFF is responsible for inhibiting osteoclast formation and bone resorption, and that NO is likely a mediator of the mechanical effects in bone leading to the inhibition of osteoclast formation and bone resorption (Chapter 5). Finally, during orthodontic force application, iNOS-positive osteocytes appeared mainly in the compression area, while in the tension area eNOS-positive osteocytes were present (Chapter 6). This suggests that eNOS mediates bone formation in the tension area, while iNOS mediates inflammation-induced bone resorption in the compression area. Both eNOS and iNOS seem to be important regulators of bone remodeling during orthodontic force application (Chapter 6). In sum, osteocytes play a key role in the bone adaptation process to mechanical loading, and this process is at least partially mediated by NO. We present a theory that explains the process of tooth movement such as occurs after orthodontic load. NO is likely a key molecule in this process, as its absence would lead to osteoclast attraction by causing osteocyte apoptosis, while its production by well-strained osteocytes leads to osteoclast withdrawal.

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CHAPTER 8

GENERAL ABSTRACT

GENERAL ABSTRACT

Bone is a living tissue and is able to alter its mass and structure to its mechanical environment. Increased mechanical loading results in a gain of bone mass and mineral density *in vivo*, while unloading of bones is known to reduce bone formation, mineral content, and bone matrix protein production. This process is known as functional adaptation of bone, and it serves to obtain bones that combine a proper resistance against mechanical failure with a minimum use of material. Adaptation of bone to changing environmental demands is obtained during the complicated process of bone remodeling. It is currently believed that the process of bone adaptation is governed by the osteocytes, which respond to the loading-induced flow of interstitial fluid through the lacuno-canalicular network by producing signaling molecules that can regulate the activity of the effector cells, the osteoclasts and the osteoblasts, which subsequently leads to adequate bone mass and structure.

This thesis examined the role of mechanical loading in various aspects of bone adaptation such as occurs during an orthodontic tooth movement, at the cellular level. Osteocyte apoptosis likely regulates bone remodeling by attracting osteoclasts. Nitric oxide (NO) is an important signaling molecule in response to mechanical loading, and is produced by osteocytes through the activity of constitutive endothelial nitric oxide synthase (eNOS) and/or inducible nitric oxide synthase (iNOS). We hypothesized that the osteocytes play a key role in the adaptation of bone to mechanical loading, and that this bone adaptation process is regulated by NO. To test this hypothesis, we addressed the following scientific questions:

1. Is there a relationship between osteocyte density, bone remodeling parameters, gender, and osteoporosis?
2. Does mechanical stimulation by fluid flow inhibit tumor necrosis factor-α-induced apoptosis of osteocytes, and is the inhibition of osteocyte apoptosis mediated by loading-induced NO production?
3. Which apoptosis-related genes alter their expression in response to

a physiological mechanical load by pulsating fluid flow in osteocytes, and is the alteration of apoptosis-related genes mediated by loading-induced NO production?

4. Are mechanically-stimulated osteocytes capable to modulate osteoclast formation and bone resorption via soluble factors such as NO?
5. Does orthodontic force stimulate constitutive eNOS and/or iNOS in rat osteocytes?

To find answers to these questions we investigated the relationship between osteocyte density, bone remodeling parameters, gender, and osteoporosis. We showed that the number of osteocytes embedded in the bone matrix depends on gender, and differs between healthy and osteoporotic subjects (Chapter 2). Osteoporotic patients showed reduced bone turnover and changed bone architecture, which is characteristic for osteoporosis, and the results are consistent with impaired osteoblast function in osteoporotic patients. The reduced osteocyte numbers in female osteoporotic patients might relate to imperfect bone remodeling leading to reduced bone mass and strength (Chapter 2).

Osteocyte apoptosis likely is the signal for osteoclast recruitment. Osteoclastic attack is directed towards apoptotic osteocytes, suggesting a key regulatory role for osteocyte apoptosis in bone remodeling, such as occurs after orthodontic load application. We studied osteocyte apoptosis induced by tumor necrosis factor-α (TNF-α) after pulsating fluid flow (PFF) application (Chapter 3). To validate that NO may modulate apoptosis, the release of NO was inhibited by N^{G}-Nitro-L-Arginine Methyl Ester (L-NAME). We found that fluid shear stress inhibits TNF-α-induced apoptosis specifically in osteocytes, but not in osteoblasts or periosteal fibroblasts. This inhibitory effect was, at least in part, mediated by NO. This suggests a regulatory role for osteocyte apoptosis in osteoclastic bone resorption during bone remodeling such as occurs after application of an orthodontic load (Chapter 3).

Which apoptosis-related genes alter their expression in response to a physiological mechanical load is unknown. We studied apoptosis-

related gene expression in response to PFF in osteocytes, osteoblasts, and fibroblasts (Chapter 4). To validate that NO may modulate apoptosis-related gene expression in osteocytes, we inhibited the release of NO by L-NAME. We measured gene expression of Bcl-2, caspase-3, p53, and c-Jun, because these molecules are key regulating molecules of cell apoptosis. We found that PFF stimulates Bcl-2 gene expression and inhibits caspase-3 gene expression, but did not alter p53 and c-Jun gene expression in osteocytes. Inhibition of NO synthesis by L-NAME prevented the PFF-mediated changes in Bcl-2 and caspase-3 gene expression in osteocytes. This suggests that NO is, at least in part, responsible for the loading-induced inhibition of osteocyte apoptosis via alterations in Bcl-2 and caspase-3 gene expression (Chapter 4).

Pericellular fluid may be critical in transmitting soluble mediators from osteocytes to other bone cells to produce a desired response to mechanical stimulation. In depth examination of the effects of conditioned medium from mechanically-stimulated osteocytes on osteoclast formation and activity can help to expand our understanding of the soluble factors released by osteocytes into the pericellular fluid. We investigated if mechanically-stimulated osteocytes are capable to modulate osteoclast formation and bone resorption via soluble factors (Chapter 5). To validate that NO may modulate osteoclast formation and bone resorption, the release of NO was inhibited by L-NAME. We found that osteocytes subjected to PFF inhibit osteoclast formation and resorption via soluble factors, and the release of these factors was at least partially dependent on activation of an NO pathway in osteocytes in response to PFF. It appeared that the osteocyte is more responsive to PFF than the osteoblast or periosteal fibroblast regarding to the production of soluble factors affecting osteoclast formation and bone resorption (Chapter 5).

Orthodontic tooth movement is characterized by sequential reactions of the periodontal tissues to biomechanical forces. We hypothesized that eNOS and iNOS regulate the tissue response to orthodontic force, and therefore we investigated eNOS and iNOS expression in osteocytes during orthodontic force application in a rat model (Chapter 6). Immunohistochemical staining revealed that in the

tension area, eNOS-positive osteocytes increased from 24 hrs on, while iNOS-positive osteocytes remained largely constant. In the compression area, iNOS-positive osteocytes increased already after 6 hrs, while eNOS-positive osteocytes increased after 24 hrs. This suggests that eNOS mediates bone formation in the tension area, while iNOS mediates inflammation-induced bone resorption in the compression area. Both eNOS and iNOS seem to regulate bone remodeling during orthodontic force application (Chapter 6).

ALGEMENE SAMENVATTING

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Bot is een levend weefsel en is in staat om zijn massa en structuur aan te passen aan de mechanische omstandigheden. Verhoogde mechanische belasting resulteert in een toename in botmassa en mineraaldichtheid *in vivo*, terwijl bekend is dat verlaagde belasting resulteert in een afname van botformatie, mineraalgehalte en botmatrix eiwit productie. Dit proces staat bekend als functionele adaptatie van bot, en op deze wijze ontstaan botten die bestand zijn tegen breken, maar die tegelijkertijd uit een minimum aan botmateriaal bestaan. Adaptatie van bot aan de veranderende mechanische belasting wordt verkregen tijdens het gecompliceerde proces van botremodellering. Men neemt tegenwoordig algemeen aan dat bot mechanische belasting signaleert middels het cellulaire netwerk van osteocyten in de botmatrix, en dat de belasting wordt waargenomen via een belasting-geïnduceerde interstitiële vloeistofstroom in het lacuno-canaliculaire netwerk rond de osteocyten. De osteocyten produceren signaalmoleculen die de activiteit van effectorcellen, de osteoclasten en osteoblasten, kunnen reguleren en dit resulteert vervolgens in een optimale botmassa en botstructuur.

Dit proefschrift onderzocht de rol van mechanische belasting op verschillende aspecten van botadaptatie op cellulair niveau, zoals gebeurt tijdens een orthodontische tandverplaatsing. Osteocyt apoptose reguleert waarschijnlijk botremodellering door osteoclasten aan te trekken. Stikstofoxide (NO) is een belangrijk signaalmolecuul dat vrijkomt als reactie op mechanische belasting. Het wordt door osteocyten geproduceerd door de activiteit van constitutief endotheel stikstofoxide synthase (eNOS) en/of door induceerbaar stikstofoxide synthase (iNOS). Onze hypothese was dat de osteocyten een belangrijke rol spelen tijdens de adaptatie van bot op mechanische belasting, en dat dit botadaptatie proces wordt gereguleerd door NO. Om deze hypothese te testen hebben we ons gericht op de volgende wetenschappelijke vragen:

1. Is er een relatie tussen osteocytdichtheid, botremodellering parameters, geslacht en osteoporose?

2. Wordt tumor necrosis factor-α-geïnduceerde apoptose in osteocyten geïnhibeerd door mechanische stimulatie van een vloeistofstroom, en wordt de inhibitie van osteocyt apoptose gemedieerd door belasting-geïnduceerde NO productie?
3. Welke apoptose-gerelateerde genen veranderen hun expressie ten gevolge van een fysiologische mechanische belasting door een pulserende vloeistofstroom in osteocyten, en wordt de verandering van de apoptose-gerelateerde genen gemedieerd door belasting-geïnduceerde NO productie?
4. Zijn mechanisch-gestimuleerde osteocyten in staat om osteoclast formatie en botresorptie te moduleren door oplosbare factoren zoals NO?
5. Stimuleert orthodontische belasting constitutief eNOS en/of induceerbaar iNOS in osteocyten in ratten?

Om antwoord te krijgen op deze vragen hebben we de relatie tussen osteocytdichtheid, botremodellering parameters, geslacht en osteoporose onderzocht. We hebben aangetoond dat het aantal osteocyten ingebed in de botmatrix afhankelijk is van het geslacht, en verschilt tussen gezonde personen en patiënten met osteoporose (Hoofdstuk 2). Bij osteoporotische patiënten werd een afname van de bot-turnover gezien en werden veranderingen van de architectuur van het bot waargenomen die kenmerkend zijn voor osteoporose. Deze bevindingen lijken te bevestigen dat bij patiënten met osteoporose de functie van osteoblasten is verstoord. Bij vrouwelijk patiënten met osteoporose kan de afname in het aantal osteocyten gerelateerd zijn aan gebrekkige botremodellering en dit leidt tot verminderde botmassa en botsterkte (Hoofdstuk 2).

Apoptose van osteocyten is waarschijnlijk het signaal voor het aantrekken van osteoclasten. Osteoclasten worden gestuurd naar apoptotische osteocyten, en dit suggereert een belangrijke regulerende rol voor osteocyt apoptose tijdens botremodellering, zoals ook gebeurt na applicatie van een orthodontische kracht. We hebben tumor necrosis factor-α-geïnduceerde osteocyt apoptose onderzocht na applicatie van een pulserende vloeistofstroom (PFF) (Hoofdstuk 3). Om aan te tonen

dat NO apoptose kan reguleren, werd het vrijkomen van NO geïnhibeerd door N^G-Nitro-L-Arginine Methyl Ester (L-NAME). We hebben aangetoond dat vloeistofschuifspanning tumor necrosis factor-α-geïnduceerde apoptose specifiek in osteocyten kan inhiberen, maar niet in osteoblasten of periostale fibroblasten. Het inhiberende effect werd gedeeltelijk gemedieerd door NO. Dit suggereert een regulerende rol voor osteocyt apoptose in osteoclastische botresorptie tijdens botremodellering zoals gebeurt na applicatie van een orthodontische kracht (Hoofdstuk 3).

Het is onbekend welke apoptose-gerelateerde genen hun expressie veranderen na een fysiologische mechanische belasting. We hebben apoptose-gerelateerde genexpressie onderzocht na PFF in osteocyten, osteoblasten en fibroblasten (Hoofdstuk 4). Om aan te tonen dat NO apoptose-gerelateerde genexpressie in osteocyten kan reguleren, hebben we het vrijkomen van NO geïnhibeerd door L-NAME. We hebben genexpressie van Bcl-2, caspase-3, p53 en c-Jun onderzocht omdat deze genen een sleutelrol spelen tijdens cel apoptose. We hebben aangetoond dat PFF Bcl-2 genexpressie stimuleert en caspase-3 genexpressie inhibeert. p53 en c-Jun genexpressie veranderde niet in osteocyten na PFF. Inhibitie van NO door L-NAME voorkwam de PFF-gemedieerde veranderingen in Bcl-2 en caspase-3 genexpressie in osteocyten. Dit suggereert dat NO gedeeltelijk verantwoordelijk is voor de belasting-geïnduceerde inhibitie van osteocyt apoptose door veranderingen in Bcl-2 en caspase-3 genexpressie (Hoofdstuk 4).

De transmissie van oplosbare factoren van osteocyten via de pericellulaire vloeistof naar andere botcellen kan essentieel zijn om een gewenste antwoord te krijgen op mechanische stimulatie. Grondig onderzoek naar de effecten van het geconditioneerde medium van mechanisch-gestimuleerde osteocyten op osteoclast formatie en activiteit draagt bij om de kennis van oplosbare factoren, vrijgegeven door osteocyten in de pericellulaire vloeistof, uit te breiden. We hebben onderzocht of mechanisch-gestimuleerde osteocyten in staat zijn om osteoclast formatie en botresorptie te moduleren via oplosbare factoren (Hoofdstuk 5). Om aan te tonen dat NO osteoclast formatie en botresorptie kan reguleren, werd het vrijkomen van NO geïnhibeerd

door L-NAME. We hebben aangetoond dat osteocyten onderworpen aan PFF osteoclast formatie en resorptie kunnen inhiberen via oplosbare factoren, en dat het vrijkomen van deze oplosbare factoren gedeeltelijk afhankelijk is van de activatie van NO in osteocyten na PFF. Het bleek dat de osteocyten gevoeliger zijn voor PFF dan osteoblasten of periostale fibroblasten wat betreft de productie van oplosbare factoren die osteoclast formatie en botresorptie beïnvloeden (Hoofdstuk 5).

Orthodontische tandverplaatsing wordt gekenmerkt door een opeenvolgende reactie van parodontale weefsels op biomechanische krachten. Onze hypothese was dat eNOS en iNOS het antwoord van de weefsels op orthodontische kracht reguleren, en daarom hebben we eNOS en iNOS expressie onderzocht in osteocyten tijdens een applicatie van een orthodontische kracht in een rattenmodel (Hoofdstuk 6). Immunohistochemische kleuring heeft aangetoond dat aan de tensiezijde de eNOS-positieve osteocyten toenamen vanaf 24 uur, terwijl de iNOS positieve osteocyten min of meer gelijk bleven. Aan de compressiezijde was er al na 6 uur een toename van de iNOS-positieve ostecyten, terwijl de eNOS-positieve osteocyten na 24 uur toenamen. Dit suggereert dat eNOS botformatie aan de tensiezijde medieert, terwijl iNOS ontsteking-geïnduceerde botresorptie aan de compressiezijde medieert. Het lijkt dat zowel eNOS als iNOS botremodellering tijdens applicatie van een orthodontische kracht reguleert (Hoofdstuk 6).

ABOUT THE AUTHOR

About the author

Djien Tan was born in Utrecht, the Netherlands on the 25th of September 1977. He grew up in Schiedam and finished his pre-university education at the Erasmiaans Gymnasium in Rotterdam. Subsequently, he studied dentistry at the Limburg University Centre (Belgium) were he obtained his bachelor's degree in 1997, and at the Catholic University Leuven (Belgium) were he obtained his master's degree in 2000. During his studies, he visited the Technical University Dresden (Germany) for an internship of the Erasmus Program.

After his study dentistry, he started working as a dentist in several general practices until August 2005. Moreover, he studied economics at the HES School of Business in Amsterdam, went to Brasil to work as a volunteer in the "Zahnärtzliches Hilfsprojekt Brasilien", and followed the "International Short-Term Course in Orthodontics" at the University of Aarhus (Denmark).

In 2002, he started his PhD project entitled "Osteocyte Apoptosis and Bone Adaptation" at the Department of Oral Cell Biology of the ACTA – UvA and VU, Research institute MOVE, under supervision of Prof. Dr. J. Klein Nulend and Prof. Dr. A.M. Kuijpers-Jagtman as promoters, and Dr. A. Bronckers and Dr. J. Maltha as co-promoters, leading to this thesis. Since August 2005, he is resident in training to become an orthodontist at the Radboud University Nijmegen Medical Centre.

Attended International Meetings

2002 78th Congress of the European Orthodontic Society, Sorrento, Italy
2003 Biology of the Tissues in Orthodontics, Brussels, Belgium
2003 81st General Session & Exhibition of the International Association for Dental Research, Göteborg, Sweden
2004 80th Congress of the European Orthodontic Society, Aarhus, Denmark
2005 83rd General Session & Exhibition of the International Association for Dental Research, Baltimore, MD, USA (poster presentation)
2005 81st Congress of the European Orthodontic Society, Amsterdam, the Netherlands (oral presentation)
2005 6th International Orthodontic Congress of the World Federation of Orthodontics, Paris, France
2006 82nd Congress of the European Orthodontic Society, Vienna, Austria (oral presentation)
2007 83rd Congress of the European Orthodontic Society, Berlin, Germany
2008 84th Congress of the European Orthodontic Society, Lisbon, Portugal (oral presentation)

Attended National Meetings

Pathophysiology of the Oral Tissues (2002)

Annual Meeting of the Netherlands Institute for Dental Science (IOT) (2003, 2004 poster presentation, 2005 oral presentation, 2006, 2007)

Annual Meeting of the Dutch Society for Calcium and Bone Metabolism (NVCB) (2003 oral presentation, 2004 oral presentation, 2005, 2007)

47th NWO Meeting for Growth and Development of Head and Neck (2004, oral presentation)

Meeting of the Skeletal Tissue Engineering Group Amsterdam (STEGA) (2005, oral presentation)

Meeting of The Dutch Association of Orthodontists (VvO) (2005, 2006, 2007 oral presentation, 2008)

Meeting of the Dutch Society for the Study of Orthodontics (NVOS) (2005, 2006, 2007, 2008)

Grants and Awards

2005 Stichting Anna Fonds, travel grant
2008 The Dutch Association of Orthodontists (VvO) Research Award

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DANKWOORD

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